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Phosphorylation sites on tau by tau protein kinase I, a bovine derived kinase generating an epitope of paired helical filaments

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Key words: Tau protein; Protein kinase; Phosphorylation; Paired helical filament; Alzheimer disease; Microtubule

Tau protein kinase I (TPKI) isolated from bovine brain has been determined to phosphorylate tau at four distinct sites by detecting modified Ser and Thr residues with protein sequencer. Ser199, Thr231, Ser396 and Ser413 were all found to have been phosphorylated by TPKI (numbering of amino acids was done in relation to the longest human tau [Neuron, 3 (1989) 519–526]). These phosphorylations generate an epitope of PHF (paired helical filaments) and eliminate the recognition of tau by the monoclonal antibody, tau-1. These results suggested that TPKI might be responsible for at least some of the phosphorylation of tau to induce PHF formation.

Paired helical filaments (PHF) accumulate in the brain of Alzheimer disease (AD) patients. The major component of PHF is tau [17, 25], a microtubule-associated protein, which is abnormally phosphorylated [8, 12, 20]. It is generally accepted that this phosphorylation triggers the PHF formation.

Anti-PHF polyclonal antibodies contained an antibody which reacted with phosphorylated tau (ptau), but not with dephosphorylated tau [12]. By following the activity which generates the ptau epitope, we purified a novel protein kinase (tau protein kinase I, TPKI) from bovine brains [15]. The epitope was not generated by other protein kinases tested so far, including cAMP-dependent protein kinase, Ca^{2+}/calmodulin kinase II and protein kinase C. Although mitogen activated protein (MAP) kinase [5, 19] and cdc2 kinase [19] were recently reported to reduce immunoreactivity with monoclonal antibody (mAb) tau-1 and generate other PHF epitopes reactive with mAbs AT8 and SMI34, TPKI was different from these kinases, judged from its amino acid sequence (unpublished data).

Previously, we detected Ser/Thr Pro kinase activity in crude TPKI fraction, and have reported the phosphorylation sites on tau by the kinase activity [14]. This activity was, however, attributed to a second protein kinase in the fraction, tau protein kinase II (TPKII) [15]. Further studies showed the significance of TPKII for phosphorylation of tau. Using antibodies against the sites phosphorylated by TPKII, it was demonstrated that tau in normal brain has already been partially phosphorylated at these sites [2]. Since the normal tau did not have the ptau epitope in PHF, the phosphorylation by TPKII is not directly related to the ptau epitope in PHF. Prior phosphorylation of tau by TPKII, however, enhanced the phosphorylation by TPKI, indicating that TPKII is important for regulation of the abnormal phosphorylation found in PHF [2]. Since TPKI could not phosphorylate synthetic non-phosphorylated peptides which we previously used for determination of the phosphorylation sites by the crude kinase fraction, we detected only phosphorylation sites by TPKII. To understand the abnormal phosphorylation, it is important to determine the phosphorylation sites by TPKI. In this report we present the sites phosphorylated by TPKI, in addition to those by TPKII.

Purification of TPKI and TPKII [15], and preparation and phosphorylation of tau [2] were described elsewhere. Although SDS-polyacrylamide gel electrophoresis of bovine tau indicated five bands of tau (Fig. 1A), each isolated band of tau [1] showed similar changes of properties after the phosphorylation; mobility shift, generation of the ptau epitope and disappearance of tau-1 epitope

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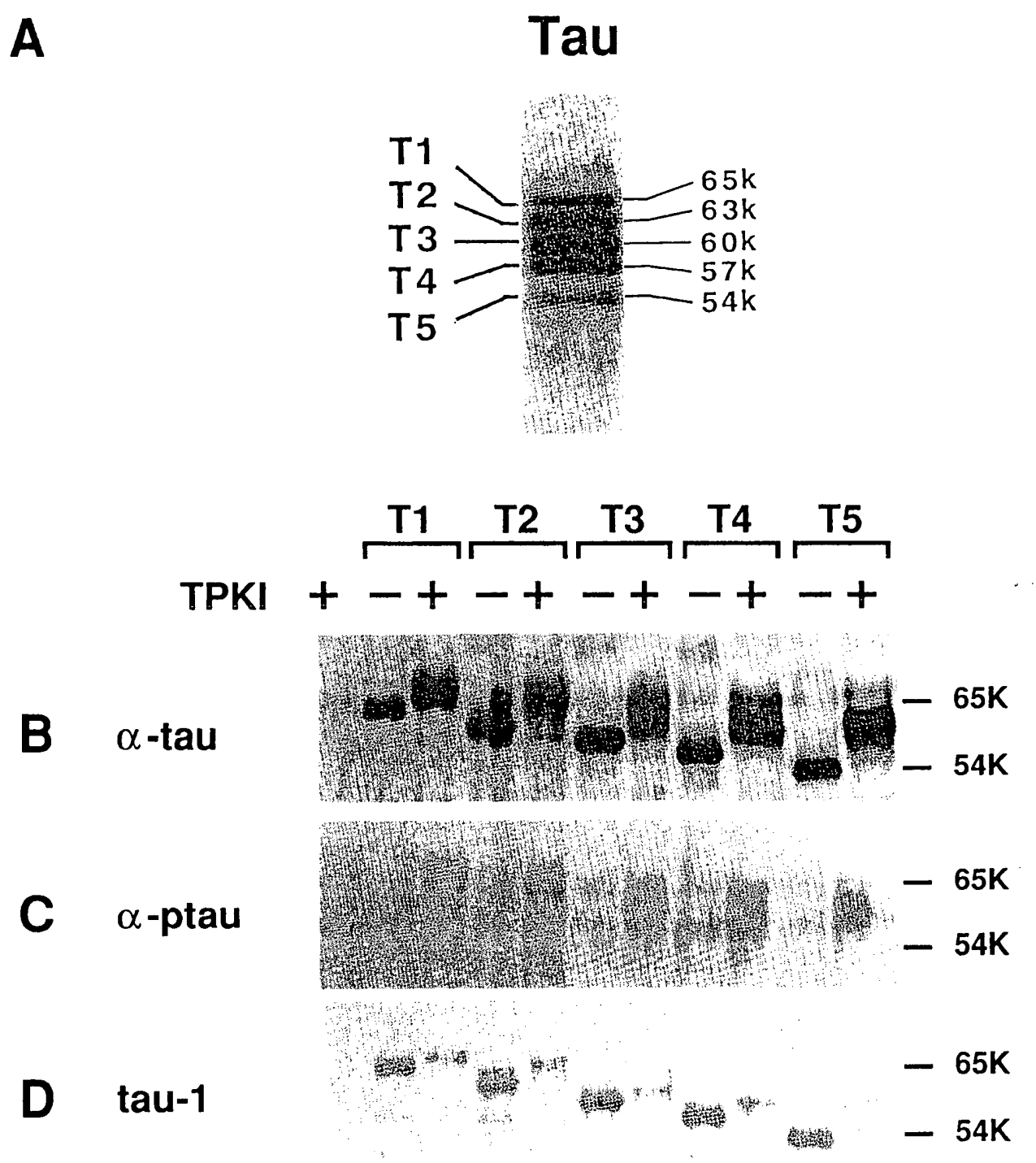

Fig. 1. Immunoblotting of each tau band with anti-ptau and tau-1. Protein staining pattern of bovine tau shows that there are five bands of tau (T1 to T5) (A). Protein from each band was extracted from the SDS–polyacrylamide gel by the method of Aizawa et al [1]. Each tau was non-treated (−) or phosphorylated (+) by TPKI, electrophoresed, transfered onto nitrocellulose membrane, and immunoblotted with anti-tau antibody (rabbit polyclonal antibody purchased from Sigma, ×1000 dilution) (B), anti-ptau from anti-PHF antiserum (a kind gift from Dr. Y. Ihara at the University of Tokyo, ×100 dilution) (C) and monoclonal antibody tau-1 (purchased from Boehringer Mannheim, ×200 dilution) (D). Immmunoblotting was done according to the standard method of Vectastain ABC system (Vector Laboratories) using 4-chloro-1-naphthol as the chromogenic substrate.

after phosphorylation by TPKI (Fig. 1B–D), suggesting that phosphorylation sites were common among the five isoforms. Therefore, we used the mixture of tau bands as the substrate for the determination of phosphorylation sites. Tau was fully phosphorylated by TPKII and cold ATP, and consequently by TPKI and [γ-^{32}P]ATP. About 4 mol of labelled phosphates were incorporated into 1 mol of tau by TPKI. Phosphopeptide mapping and de-

termination of phosphorylation sites were performed by our method reported previously [14]. Three radioactive peptides were obtained from digest of the phosphorylated tau by endoproteinase Lys-C. These peptides were identical to those previously designated by us as K1, K2 and K3 [14]. When compared with the amino acid sequence of the longest human tau [7], the sequences of K1, K2 and K3 were found in the regions of 226–240, 191–224 and 396–438, respectively. The amino acid sequences of these regions are common between bovine tau and human tau. All tau isoforms have these regions [7, 11]. About half (40%) of peptide eluting at the position of K3 did not start at Ser396 but at Ala384 (Fig. 2). Radioactivity ratio of K1:K2:K3 was 0.5:1:2. Phosphorylation sites were determined using pulse-liquid phase protein sequencer by detecting serine (or threonine) residues yielding no PTH-serine (or PTH-threonine) after phosphorylation by TPKI.

The sequencing studies showed that TPKI phosphorylated Ser199, Thr231, Ser396 and Ser413 (corresponding to Ser141, Thr173, Ser307 and Ser324 in the shortest human tau [6]) (Fig. 2). All of the phosphorylation sites on tau by TPKI were located near the sites phosphorylated by TPKII. Considering the fact that the phosphorylation sites by TPKI are adjacent to the TPKII-reactive sites, TPKI may recognize an altered conformation of tau resulting from the phosphorylation by TPKII.

Two forms of peptide K3 were observed; one with phosphate at Ser396 (K3 starting at Ala384) and one with unmodified Ser396 (K3 starting at Ser396). This result indicated that endoproteinase Lys-C could cleave at Lys395 only if Ser396 were not phosphorylated. In fact, phosphorylation of K3 was detected only on the peptides starting with Ala384, not the one starting with Ser396. Ser396 has also been observed to be phosphorylated in a KSPV sequence in A68, a class of PHF [20].

Ser199 was located at the central position of the tau-1 region recognized by monoclonal antibody tau-1. The phosphorylation of tau by TPKI induced the disappearance of immunoreactivity with tau-1. Phosphorylation by TPKII also weakened the immunoreactivity, but to a lesser extent than that found in the case of TPKI [15]. TPKII phosphorylated Ser202 and Thr205 in the region, whereas TPKI phosphorylated Ser199. These results indicate that phosphorylations of Ser202 and Thr205 were not sufficient for the disappearance of tau-1 epitope, and that the disappearance was enhanced by phosphorylation of Ser199 in addition of phosphorylation of Ser202 and Thr205. Another group also reported that phosphorylation of Ser199 and Ser202 were detected as soon as tau could not react with tau-1 [3, 9].

Ser413 was located in the ptau region recognized by anti-ptau prepared from anti-PHF antiserum. This region also contains Ser404 which was phosphorylated by TPKII. It was reported that phosphorylation of Ser404 was necessary for formation of the ptau epitope [16]. In our experiment, however, Ser404 was already phosphorylated in normal tau having no PHF epitope [2]. Our results indicated that the ptau epitope was generated by phosphorylation of Ser413, but not by that of Ser404. To confirm this, we synthesized by our procedure [22] two phosphopeptides named PS413 and PS404. PS413 spans amino acids number 408 to 418 containing a phosphate at Ser413. PS404 spans amino acids number 395 to 406 containing a phosphate at Ser404. Enzyme linked immunoadsorbent assay [2] showed that the anti-ptau antibody bound to PS413 but not to the non-phosphorylated counterpart or PS404. Moreover, an antibody raised against PS413 bound to tau phosphorylated by TPKI.

These phosphorylation sites by TPKI and TPKII were not in the tubulin-binding regions [4]. The phosphorylation by TPKI and TPKII, however, diminished the ability of tau promoting microtubule formation [24] (Fig. 3), indicating that the regions posessing these phosphorylation sites have an ability to regulate tubulin-binding. This fact was supported by an observation that these regions enhanced affinity of tau to tubulin [4].

Another group determined phosphorylation sites in PHF-tau prepared from Sarkosyl-insoluble pellet of AD brain homogenate [10]. The phosphorylation sites were Thr231, Ser235, Ser262, and more than two sites in each of the tau-1 region (residues 191–225) and the most car-

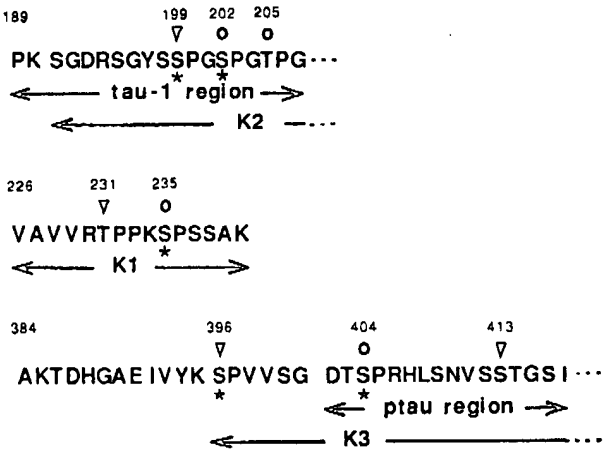

Fig. 2. Sites on tau phosphorylated by TPKI and TPKII. The amino acid numbering is expressed in accordance to that of the longest human tau [7]. Arrowheads indicate the phosphorylation sites on tau protein by TPKI. Open circles indicate the phosphorylation sites by TPKII. Tau-1 region [18] and ptau [16] region are also indicated. Asterisks indicate phosphorylation sites by a kinase activity thought to be MAP kinase (ERK2) [9].

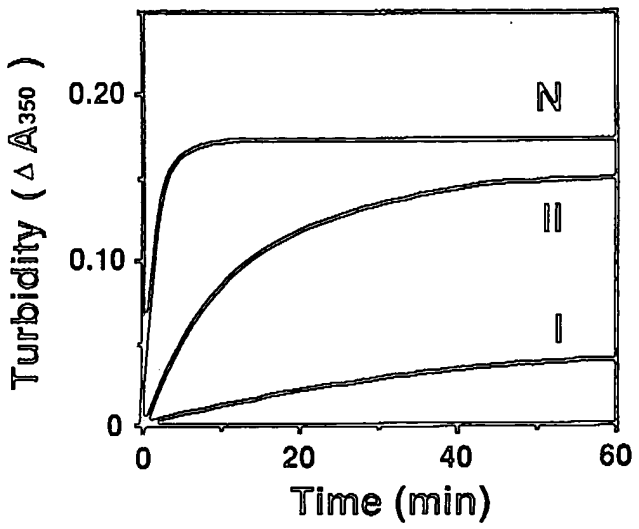

Fig. 3. Inhibition of microtubule formation by the phosphorylation of tau. Two mg/ml of tubulin was mixed with 0.2 mg/ml of normal tau (N), tau phosphorylated by TPKI (I) or tau phosphorylated by TPKII (II) in ice-cold buffer solution containing 0.1 M MES (pH 6.5), 0.5 mM magnesium acetate, 1 mM EGTA, 5 mM mercaptoethanol, 10% glycerol and 0.5 mM GTP. After mixing, the samples were incubated at 37°C. Microtubule formation was monitored by measurement of turbidity at 350 nm.

boxyl-terminal portion (residues 386–438). These sites contained the phosphorylation sites by TPKI and TPKII. TPKII sites were already phosphorylated in normal tau [2]. These facts suggested that TPKI is a possible candidate for protein kinase(s) phosphorylating tau to induce PHF-tau.

Interestingly, the phosphorylation sites of TPKI and TPKII were almost coincident with those necessary for transformation of tau to PHF-tau by a so-called MAP kinase [9]. But there were minor differences. MAP kinase phosphorylated Ser46 and Ser422, but these phosphorylations were not detected in the case of TPKI and TPKII. Ser413 was phosphorylated by TPKI but not by the MAP kinase. Thr231, one of phosphorylation sites in the PHF-tau from AD brain [10], was phosphorylated by TPKI but not by the MAP kinase. Some of phosphorylation sites by the MAP kinase were already phosphorylated in normal tau probably by TPKII [2]. Here, an important question arises; which kinase actually works in AD brain. In order to answer the question, further studies are necessary.

Recently, two reports [13, 23] have been published on other protein kinases related to the phosphorylation of PHF-tau, including one describing protein kinase activity isolated by immunoaffinity chromatography using a PHF-specific antibody as a ligand [23]. This kinase was shown to be inhibited completely by hemin at 10 μM. We found that TPKI was also sensitive to inhibition by hemin but it required more hemin to achieve half maximal inhibition (50 μM). An association of the protein kinase purified by immunoaffinity chromatography could result from association of the kinase with microtubules bound to tau [13]. Alternatively, TPKI might be related to this PHF-associated kinase activity. Further studies will be needed to clarify this point.

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ANTI-PHOSPHOTYROSINE AND ANTI-PHOSPHOSERINE ANTIBODIES IN SLE SERA

BY

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By using an ELISA method, we identified antiPTyr and antiPSer antibodies in the sera of some SLE patients. Afterwards, antiPTyr and antiPSer antibodies were purified by affinity chromatography on phosphotyramine-CNBr-Sepharosec olumn and on phosphoethanolamine-CNBr-Sepharose, respectively, and the specificity of the purified antibodies was demonstrated by inhibition assays. The study pointed out a higher incidence of antiPTyr than anti PSer antibodies in the sera of these patients, which suggests that some „autoantigens" from membrane might be involved.

Phosphorylation of proteins as the event of signals transduction has constituted in the last years a very interesting field for biochemists and immunologists.

Today, it is generally accepted that a phosphorylation process carried out by protein kinases by covalently modifying the cellular proteins at the serine, threonine and thyrosine level is a physiological one if it is reversible. It has been proved that the dephosphorylation is achieved in the presence of some enzymes with pleiotropic activity, called phosphoprotein phosphatases.

The existence of an equilibrium between the kinases and phosphatases activities is shown at the cellular level by the phosphorylation status of the proteins.

It has been demonstrated (1) that in physiological conditions the various tissues of the body contain different percentages of phosphoaminoacids, that the phosphoserine and phosphothreonine content is, generally, a hundred times higher than the phosphotyrosine content. But, in some pathological conditions (malignancy, viral infections), the level of phosphorylated proteins — especially on tyrosine —is significantly increased, showing a hyperactivation of the kinases (2, 3, 4). Moreover, OHTSUKA (5) pointed out the presence of anti-phosphotyrosine (antiPTyr) antibodies

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in the sera of patients with malignant and cerebrovascular diseasess Although the antigens that determine the production of these antibodie. are not known as yet it is possible that tyrosine phosphorylated proteins might be involved.

Starting with these observations we initiated a study for the identification of antiPTyr and anti-phosphoserine (antiPSer) antibodies in the sera of SLE patients. This research is also based on the fact that SLE, a disease with complex etiology, is characterized by the uncontrolled production of autoantibodies against various nuclear components (DNA, RNA, histones) (6, 7, 8, 9), cytoskeleton (vimentin) (10) and plasma membrane, especially against antigens on the T lymphocytes membranes (11). Several of these proteins could be transitoryy phosphorylated following the cellular activation, thus determining the appearance of these autoantibodies.

MATERIALS AND METHODS

Human sera: We used 23 sera from SLE patients in an active stage of the disease and 30 sera from healthy donors.

ELISA for antiPTyr and antiPSer antibodies in human sera

As antigens, we used phosphotyrosine, respectively phosphoserine, coupled to bovine serum albumin (PTyr—BSA, PSer—BSA) with a method described (12).

The polystyrene plates were covered with PSer—BSA and respectively with PTyr—BSA at 100 μg/ml in PBS. After overnight incubation at the room temperature, the plates were washed with PBS containing 0.02 % Tween 20 (PBS—T) and then the human sera diluted 1/100 in PBS—T containing 1 % bovine serum albumin (PBS—T—BSA) was added and incubated one hour at 37°C. After washing, the plates were covered with anti-human IgG — alkaline phosphatase conjugate for an hour at 37°C.

The reaction is detected with p-nitrophenyl phosphate. The results are expressed as optical density (OD).

ELISA inhibition

The human sera purified antibodies, diluted at 50 μg/ml, are preincubated for one hour at 37°C with 10mM PTyr, PSer and respectively BSA and afterwards they are used in the above described ELISA. The inhibition percentage is expressed as follows:

$$\frac{OD\ noninhibited\ Ab - OD\ inhibited\ Ab}{OD\ noninhibited\ Ab} \times 100$$

The purification of antiPTyr and antiPSer antibodies by affinity chromatography

The purification on antiPTyr antibodies on a Phosphotyramine — CNBr-Sepharose column was achieved after a method described (12) for the purification of antiPTyr rabbit antibodies. For the purification of antiPSer antibodies we prepared a phosphoethanolamine-— CNBr-Sepharose column. The elution of antiPSer antibodies from the column was carried out at 30°C with 40 mM phosphoserine.

RESULTS

The study comprised the sera from 23 patients with SLE in an active stage of the disease. In order to establish the cut off (OD + 2DS) we determined the antiPTyr and antiPSer antibodies in the sera of 30 healthy donors. Thus, in the ELISA for the determination of antiPTyr

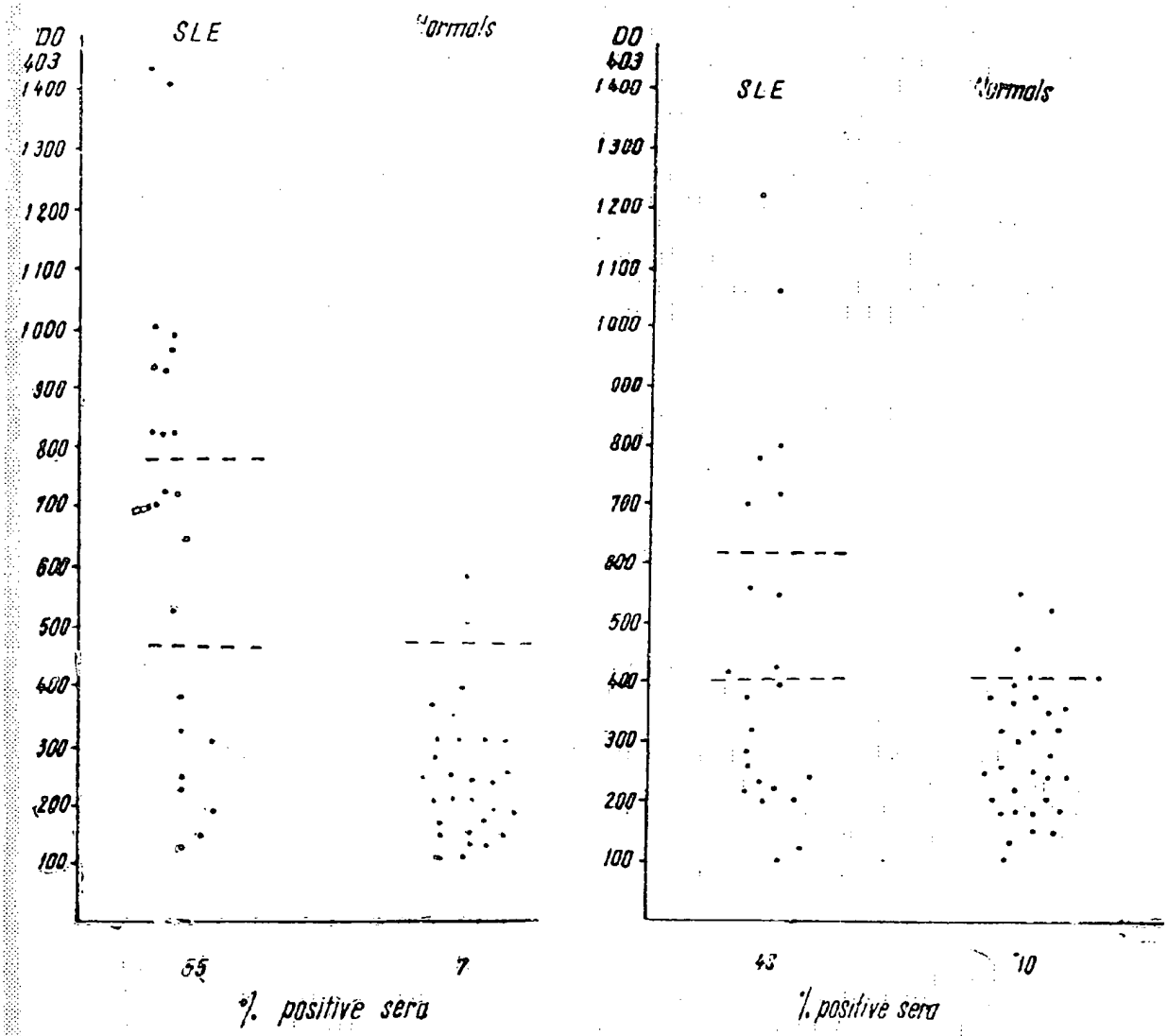

Fig. 1a — The level of antiPTyr antibodies
Fig. 1b — The level of antiPSer antibodies

antibodies the cut off was 0.470 and in the ELISA for the determination of antiPSer antibodies the cut off was 0.410.

Fig. 1 presents the level of antiPTyr (a) and antiPSer (b) antibodies in the sera of SLE patients and healthy donors.

As one can see in Fig. 1, in the healthy donors sera, the antiPTyr antibodies are present only in 7% of the cases and the antiPSer antibodies only in 10% of the cases. Therefore, the antibodies in healthy donors are presented only in a few cases and at low titres, the values that surpass the cut off being lower than 0.600. The OD mean value for antiPTyr antibodies is 0.230 and for antiPSer antibodies is 0.188.

In the SLE patients antiPTyr and antiPSer antibodies appear frequently and at high titres. In the case of antiPTyr antibodies 65% of the patients have OD values higher than the cut off — the mean of these values being about 0.900. We encounter a similar behaviour for antiPSer antibodies, 43% of the patients having OD values higher than the cut off with the mean of these values about 0.750.

In order to verify the specificity of the ELISA tested antibodies they were purified using affinity chromatography. We firstly isolated antiPTyr antibodies on phosphotyramine-Sepharose and then the anti PSer antibodies on phosphoethanolamine-Sepharose. The purified antibodies were inhibited with PTyr, PSer and, respectively, BSA and then retested with ELISA. The inhibition percentage is shown in Table 1.

Table 1

The inhibition percentage of antiPTyr and antiPSer antibodies with PTyr, PSer and BSA

Inhibitor	*Inhibition %*	
	AntiPTyr Abs	*antiPSer Abs.*
PTyr	93	5
PSer	4	95
BSA	0	0

As one can see, the antibodies purified on phosphotyramine-Sepharose column recognize exclusively PTyr (93% inhibition) and almost not at all PSer and BSA .

The antibodies eluted with phosphoserine from phosphoethanolamine-Sepharose column have specificity only for phosphoserine (95% inhibition) and not for phosphotyrosine (5% inhibition) or bovine serum albumin (0% inhibition).

CONCLUSIONS

The present work demonstrated the presence in SLE sera of anti PTyr antibodies, already pointed out before in other diseases. Moreover, this study also identifies, for the first time, the antiPSer antibodies, in the sera of these patients.

The comparative analysis of the sera from SLE patients and from healthy donors demonstrates the presence of antiPTyr and antiPSer in both cases. It is remarkable that the normal people have low titres of antibodies and only 7% are positive for antiPTyr and 10% for antiPSer. Unlike the normals, at the SLE patients, the incidence of antiPTyr-antiPSer antibodies is much higher (65%/43%), their titres being significantly higher.

The fact that the antiPTyr antibodies have a higher incidence than the antiPSer antibodies suggests that some antigens from the plasma membrane might be involved. The supposition is also sustained by the previous discovery of anti-lymphocyte plasma membrane in SLE (11). Generally, in SLE, there is a cellular hyperactivation that certainly

leads to the activation of some enzymes involved in the signals transmission. These enzymes catalyses a cascade of phosphorylation-dephosphorylation reactions of the proteins that lead the signal to the nucleus. The tyrosine phosphorylation takes place, in general, on the membrane proteins or in the immediate vicinity of these ones — proteins that can be liberated into the circulation by cellular destructions frequently met in SLE — thus becoming the target of a humoral immune response.

The fact that in SLE the antiPTyr antibodies appear more frequently and at higher titres than antiPSer could be also due to an increased immunogenicity of tyrosine as compared with the one of serine.

At present, it is difficult to specify the origin of the phosphorylated proteins that induce the immune response but it is certain that these antibodies recognize a single epitope (PTyr and PSer) present on different phosphorylated proteins.

The physiopathological involvement of these antibodies cannot be demonstrated as yet, subsequent studies being needed in order to be able to elucidate this aspect. At the same time, of great importance is the identification of the autoantigens and the study of the mechanisms by which they are generated.

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LES ANTICORPS ANTI-PHOSPHOTYROSINE ET ANTI-PHOSPHOSÉRINE DANS LE SÉRUM DE QUELQUES PATIENTS À SLE

RÉSUMÉ

Par l'utilisation de la méthode ELISA, on a identifié les anticorps anti-PTyr et anti-PSer du sérum de quelques malades de SLE. Ces anticorps (anti-PTyr et anti-PSer) ont été ensuite purifiés par chromatographie d'affinité sur une colonne de phosphotyramine CNBr—Sépharose et, respectivement, phosphoéthanolamine-CNBr-Sépharose : la spécificité des anticorps purifiés a été démontrée per des tests d'inhibition. L'étude a mis en évidence un pourcentage plus grand d'anticorps anti-PTyr que celui des anticorps anti—PSer dans les sérums de ces patients ; ce fait suggère une implication potentielle de quelques « auto-antigènes » de la membrane.

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Calponin and SM 22 isoforms in avian and mammalian smooth muscle

Absence of phosphorylation *in vivo*

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Calponin is a basic smooth-muscle-specific protein capable of binding to F-actin, tropomyosin and calmodulin *in vitro*. Using two-dimensional gel electrophoresis, we show that calponin exists as multiple isoelectric variants in avian and mammalian tissues. During chick embryogenesis, one isoform is expressed in gizzard that shows a pI identical to the most basic adult α variant; around 10 d after hatching multiple isoforms then appear. SM 22 [Pearlstone, J. R., Weber, M., Lees-Miller, J. P., Carpenter, M. R. & Smillie, L. B. (1987) *J. Biol. Chem. 262*, 5985–5991], which has sequence motifs related to calponin, displays a similar isoform pattern during development; one isoform (α) is present in the embryo and three in the adult.

In living smooth-muscle strips from chicken gizzard and guinea pig taenia coli, labelled with $^{32}PO_4$, no phosphate incorporation could be detected in any of the calponin or SM 22 isoforms during either contraction or relaxation. From the additional observation that antibodies against phosphoserine also failed to label calponin and SM 22 in two-dimensional gel immunoblots, we conclude that the multiple isoforms do not arise via differential phosphorylation. These results support the claim [Barany, M., Rokolya, A. & Barany, K. (1991) *FEBS Lett. 279*, 65–68] that calponin phosphorylation is not involved in smooth muscle regulation *in vivo*, as has been suggested from *in vitro* studies [Winder, S. J. & Walsh, M. J. (1990) *J. Biol. Chem. 265*, 10148–10155]. *In vitro* translation of porcine and chicken smooth-muscle mRNA produced only a single (α) isoform of calponin, suggesting that the adult isoforms do not derive from multiple gene products; in the same assay two polypeptides appeared in the position of SM 22, one corresponding to the α isoform and a second more basic spot, not observed in tissue samples.

Whereas calponin and SM 22 appear synchronously during smooth muscle differentiation *in vivo*, SM 22 is not fully down-regulated like calponin, metavinculin and heavy-caldesmon in smooth muscle cells in culture, pointing to a differential regulation of expression of the α SM 22 isoform during smooth-muscle phenotype modulation *in vitro*.

Smooth muscle differentiation is characterized by the expression of a family of cytoskeletal and contractile proteins, among them specific actin and myosin isoforms [1–3], desmin [4], metavinculin [5, 6] and the higher-molecular-mass variant of caldesmon [7]. Two basic proteins, conspicious only in two-dimensional gels, are also strongly and specifically expressed in smooth muscle tissue, namely calponin [8] and SM 22 [9]. Calponin has recently received much attention since current data suggest that this protein may reside on the actin thin filaments and serve as a modulator of actomyosin ATPase [9a; reviewed in 12 and 13]. The mechanism of actin-linked regulation via calponin is, however, by no means clear, either with regard to the possible role of phosphorylation [9a, 10] or with respect to the reported additional involvement of caldesmon [14]. Since SM 22 could not be shown to bind to any contractile proteins with significant affinity [9, 15], not even a tentative role could be ascribed to this protein. It was noteworthy, however, that independent sequence analysis of calponin and SM 22 revealed high similarity between extensive regions of these two proteins [9, 16, 17]. Thus, calponin and SM 22 may share some properties in common that have yet to be revealed.

On the basis of the relatedness of calponin and SM 22, we have studied their expression in parallel during smooth muscle differentiation. The results reveal interesting similarities and differences in isoform expression during smooth muscle development *in vivo* and phenotype modulation *in vitro*. In addition, evidence is furnished for the lack of calponin (and SM 22) phosphorylation in living, contracting and relaxed smooth muscle, in support of recent suggestions [18] that calponin phosphorylation is an *in vitro* phenomenon unrelated to regulation *in vivo*.

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MATERIALS AND METHODS

Protein purification

Calponin from chicken gizzard and porcine stomach smooth muscle were purified as described [16].

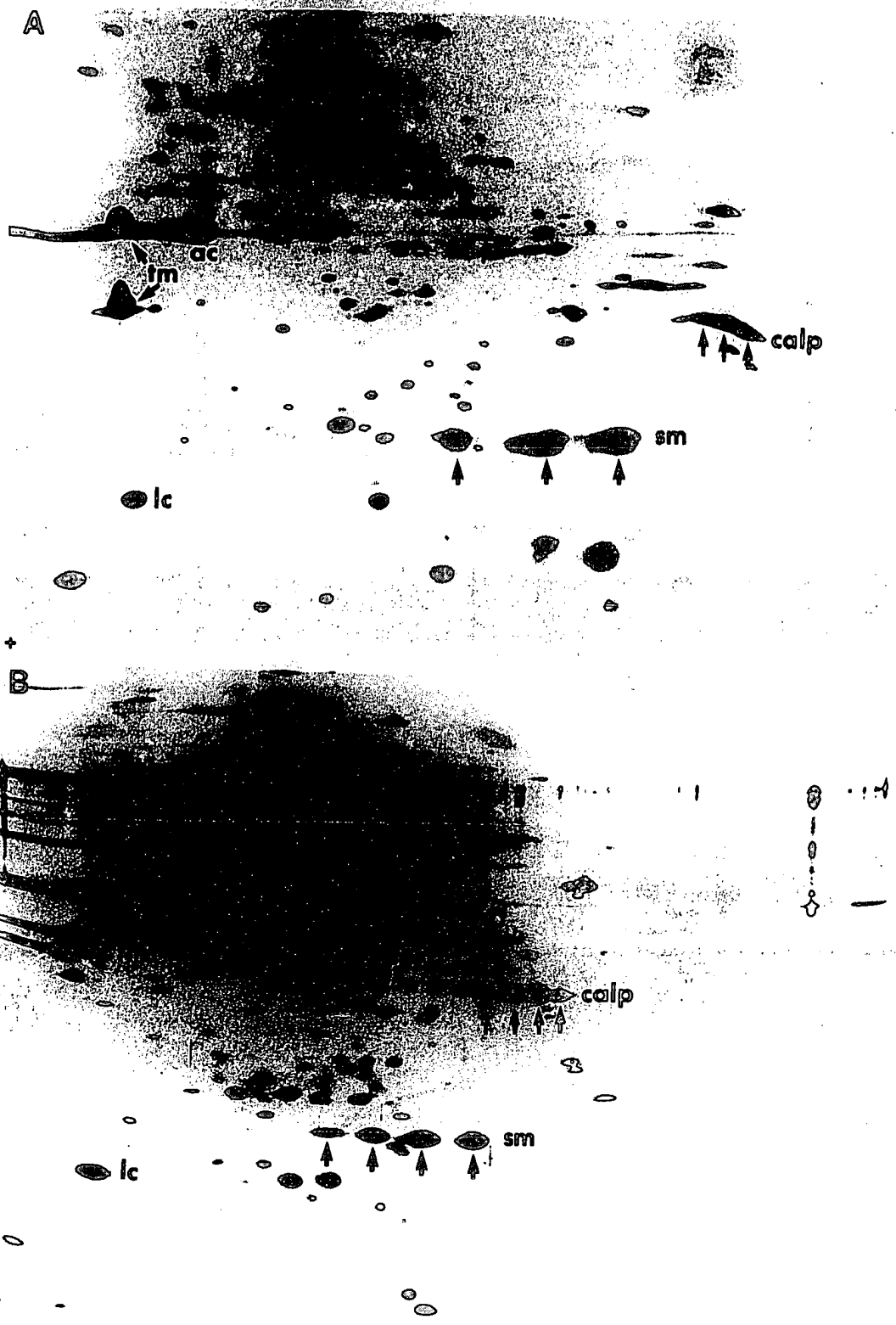


Fig. 1. Two-dimensional, silver-stained gel showing calponin (calp) and SM 22 (sm) as major proteins in chicken gizzard (A) and pig stomach (B) smooth muscle. Ac, actin; tm, tropomyosin; lc, 20-kDa myosin light chain.

Electrophoresis

Analytical gel electrophoresis was carried out on 8–22% gradient acrylamide minislab gels according to the procedure of Matsudaira and Burgess [19] in the buffer system of Laemmli [20]. Protein samples were supplemented with SDS sample buffer (final concentrations: 2.5% SDS, 1% 2-mercaptoethanol, 7% glycerol, 0.001% Bromophenol blue, 62.2 mM Tris/HCl, pH 6.8) and boiled for 1 min at 100 °C.

Two-dimensional gel electrophoresis

Two-dimensional gels were run according to the method of O'Farrell [21] using the mini two-dimensional system of Bio-Rad. First-dimension gels were run at 1000 Vh containing 2% (final concentration) Ampholines (Serva) covering pH ranges 3–10, 3–6, 5–8 and 7–9. The second dimension was performed using 15% polyacrylamide gels (dimensions 11 cm × 8 cm; 0.75 mm spacers in a Bio-Rad Mini-Protean II system) according to the same procedure employed for minislab gels (see above). Gels were silver stained according to a procedure given by Heukeshoven and Dernick [22] with minor modifications.

Sample preparation for two-dimensional gel electrophoresis

Freshly-dissected muscle pieces of 20–50 mg were frozen in liquid nitrogen, then shattered by hammering between a liquid-nitrogen-cooled metal stud and plate. Crushed material was taken up and dissolved in a tenfold volume of lysis buffer (containing 9.8 M urea and ampholines pH 7–9; 1% final concentration). 5 µl were applied on the first-dimension gels.

Immunoblotting

Western blotting of polyacrylamide gels onto nitrocellulose sheets was carried out according to Towbin et al. [23]. Silver-enhanced immunogold staining was performed following the procedure described by Moeremans et al. [24] using a secondary antibody with a gold tag (Amersham, UK). Rabbit polyclonal antibodies raised against calponin labelled both calponin and SM 22 in immunoblots (see Results). Fractions specific for calponin alone or SM 22 and calponin were purified on affinity columns with the respective proteins coupled to activated CNBr-activated Sepharose (Pharmacia, Sweden) according to the manufacturer's guidelines. The monoclonal anti-calponin antibody (CP-93) was obtained from Sigma (Sigma, St Louis, USA) and the monoclonal anti-phosphoserine antibody was kindly donated by Prof. B. Geiger (Weizmann Institute, Israel).

In vivo radiolabelling

Gizzard muscle strips were teased from thin slices of freshly dissected chicken gizzards obtained by cutting the gizzard radially with a razor blade. The strips, 1–1.5 mm wide and around 10 mg, were placed in Krebs solution containing 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 15 mM $NaHCO_3$, 2 mM $CaCl_2$, 0.1 mM $NaH_2PO_4^-$, 10 mM glucose and 2 mM NaMops (morpholinic acid titrated with NaOH to pH 7) and gassed continuously with 95% O_2/5% CO_2 (final pH 7.4). For measurements of isotonic shortening, strips were mounted in an organ bath with one end fixed and the other end attached via a thread to an Ugo Basile (Milan, Italy) isotonic transducer

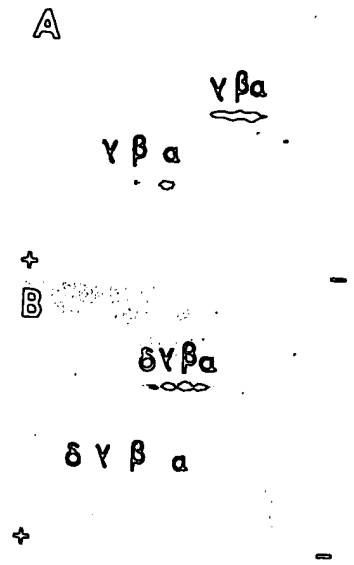


Fig. 2. Immunoblots of two-dimensional gels of chicken gizzard (A) and pig stomach (B) whole muscle samples with the calponin/SM 22 cross-reactive antibody.

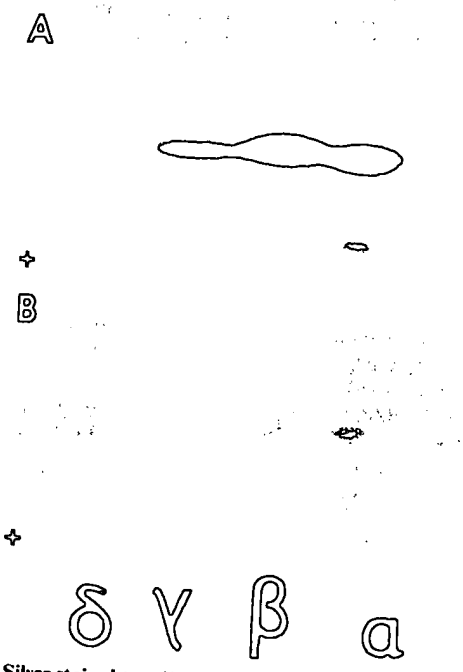


Fig. 3. Silver-stained two-dimensional gels of purified calponin of avian (A) and porcine (B) origin showing three and four isoforms, respectively.

7006. After equilibration for 90 min at a load of 0.6 g, a concentration-response curve to carbachol was obtained and responses also elicited with a depolarizing solution in which the NaCl in the Krebs solution was replaced by KCl. Strips of guinea pig taenia coli were treated in a similar manner to those from chicken gizzard.

Phosphate labelling was performed using parallel muscle strips that were not mounted on the transducer. Instead, a

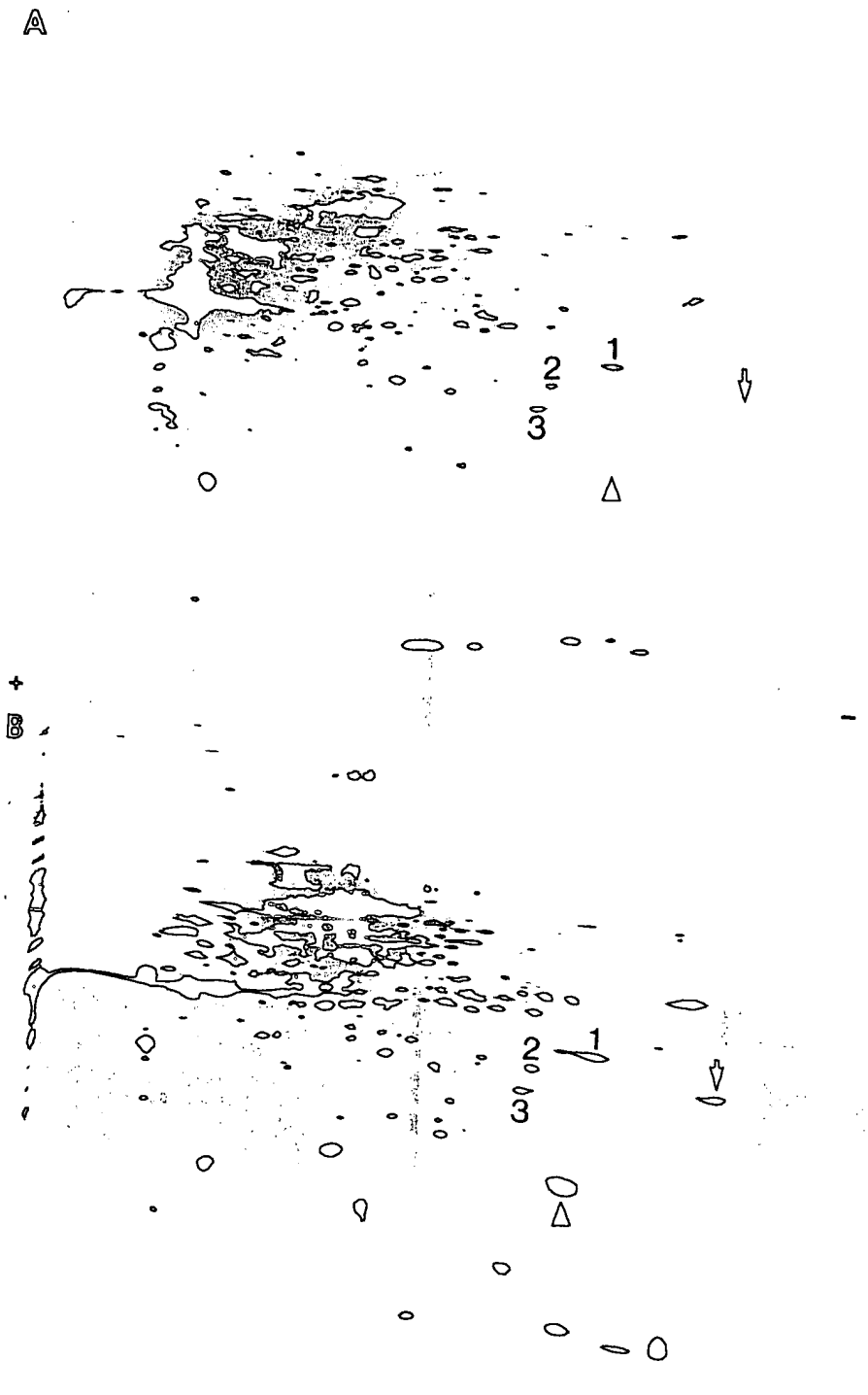


Fig. 4. Appearance of calponin and SM 22 during chick embryogenesis. (A) Whole gizzard of 7 d embryo; (B) gizzard muscle layer from a 10-d-old embryo. Arrow and arrowhead mark the positions of calponin and SM 22, respectively. 1, 2 and 3 are arbitrarily-chosen polypeptides used as landmarks on the gels. Gels are silver stained.

glass bead of 0.6 g was attached at one end of each strip using a fine stainless-steel hook. The upper end carried a similar hook that was hung on a wire crossing the top of the organ bath. The strips were incubated in Krebs solution with carrier free $^{32}PO_4^{3-}$ as $H_3PO_4^{3-}$ at 100 mCi/μl and the strips equilibrated for 90 min. Three control strips were then removed and snap-frozen in liquid nitrogen. Other strips were then contracted in solutions of the same specific radioactivity con-

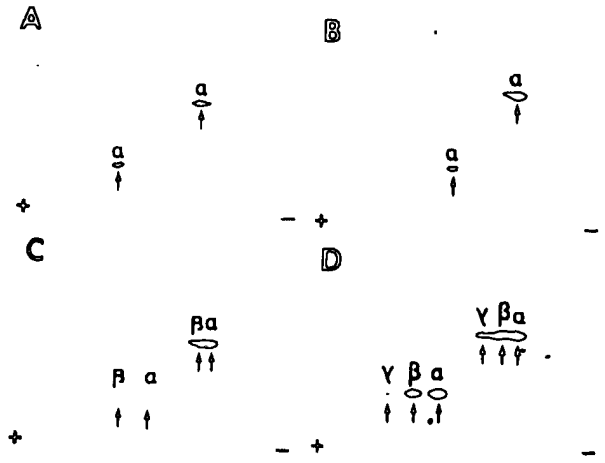


Fig. 5. Immunoblots of two-dimensional gels of gizzards taken from chicks at various embryonic and post hatching stages using calponin/SM 22 cross-reactive antibody. (A) 10-d-old embryo; (B) 19-d-old embryo; (C) 10-d-old chick (post hatching) and (D) adult (21-week-old) chicken. Note aquisition of two isoforms at post hatching d 10 (C). All isoforms are expressed in the adult (D).

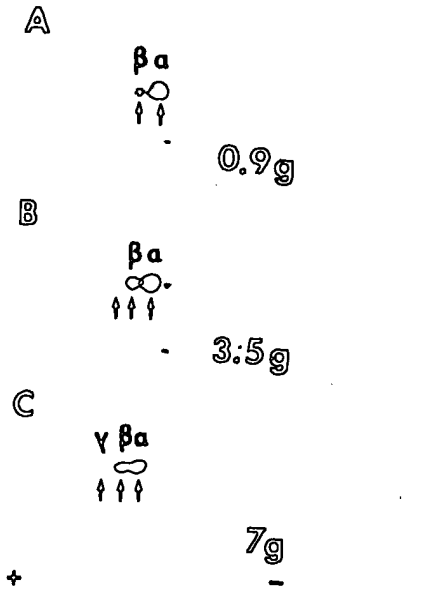


Fig. 6. Two-dimensional gel immunoblots showing the expression of calponin isoforms in pig embryos with stages given in grams. (A) Whole embryo, 0.9 g; (B) intestine of 3.5-g embryo; (C) intestine of 7-g embryo. Polyclonal monospecific calponin antibody.

taining 10 μM carbachol (to produce 96% of maximum shortening) or the KCl depolarizing solution and were removed at various times during contraction or relaxation cycles and snap-frozen. The contraction could be easily observed from the lifting of the glass bead as the strip shortened to less than 50% of its initial length. Samples were prepared for two-dimensional gel electrophoretic analysis as above. Autoradiography of the dried gels was carried out with Kodak X-AR 5 film using an exposure time of 4 d.

Primary cultures

Primary cultures of smooth muscle were obtained by dispersing gizzards of 16-d-old chick embryos with 3 mg/ml collagenase (Sigma type V) in Dulbecco's modification of Eagle's medium for 30 min at 37 C. The cells were maintained in Dulbecco's modification of Eagle's medium (Gibco) supplemented with either 2% or 10% chicken serum (Sebak, FRG), at 37 C in the presence of 5% CO_2.

Extraction and purification of poly(A) mRNA

For isolation of mRNA from pig stomach and chicken gizzard smooth muscle, a procedure according to Chirgwin et al. [25] with slight modifications was employed. 5 – 10 g tissue were frozen in liquid nitrogen and homogenized on ice in 50 – 100 ml 4.2 M guanidinium thiocyanate containing 0.1 M sodium acetate, pH 7.0 and 1% 2-mercaptoethanol using an Ultra turrax tissue homogenizer (IKA Instruments, FRG). The suspension was briefly heated to 65 C and subsequently centrifuged in a Sorvall HB4 rotor for 15 min, at 10000 rpm. The supernatant was passed through a G 22 needle several times and extracted as described [26, 27]. Poly(A) mRNA was purified by oligo(dT) column chromatography.

In vitro translation of smooth muscle mRNA

mRNA purified from adult chicken gizzard or pig stomach smooth muscle was translated in either a wheat-germ or a reticulocyte *in vitro* translation system (Promega Corporation, Madison, USA) according to the manufacturer's descriptions using 5 – 10 μg poly(A) mRNA.

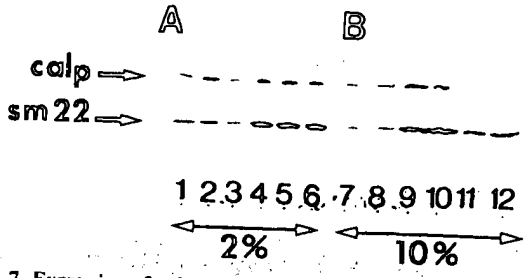


Fig. 7. Expression of calponin and SM 22 in primary and secondary cultures of chicken gizzard cells as shown by immunoblotting of a single-dimension minislab gel with calponin/SM 22 cross-reactive antibody. (A) Cells maintained in 2% serum for 1, 3 h; 2, 1 d; 3, 2 d; 4, 3 d; 5, 4 d; 6, 6 d. (B) Cells maintained in 10% serum and monitored respectively for 7, 3 h; 8, 1 d; 9, 2 d; 10, 3 d in primary culture. 11 and 12, secondary cultures of chicken gizzard cells replated after 7 d and 14 d, respectively and sampled after a further 2 d. Note loss of calponin but retention of SM 22 in secondary cultures. Variations in total amounts of calponin are related to density differences in the cell-culture dishes. Calp, calponin; sm, SM 22.

RESULTS

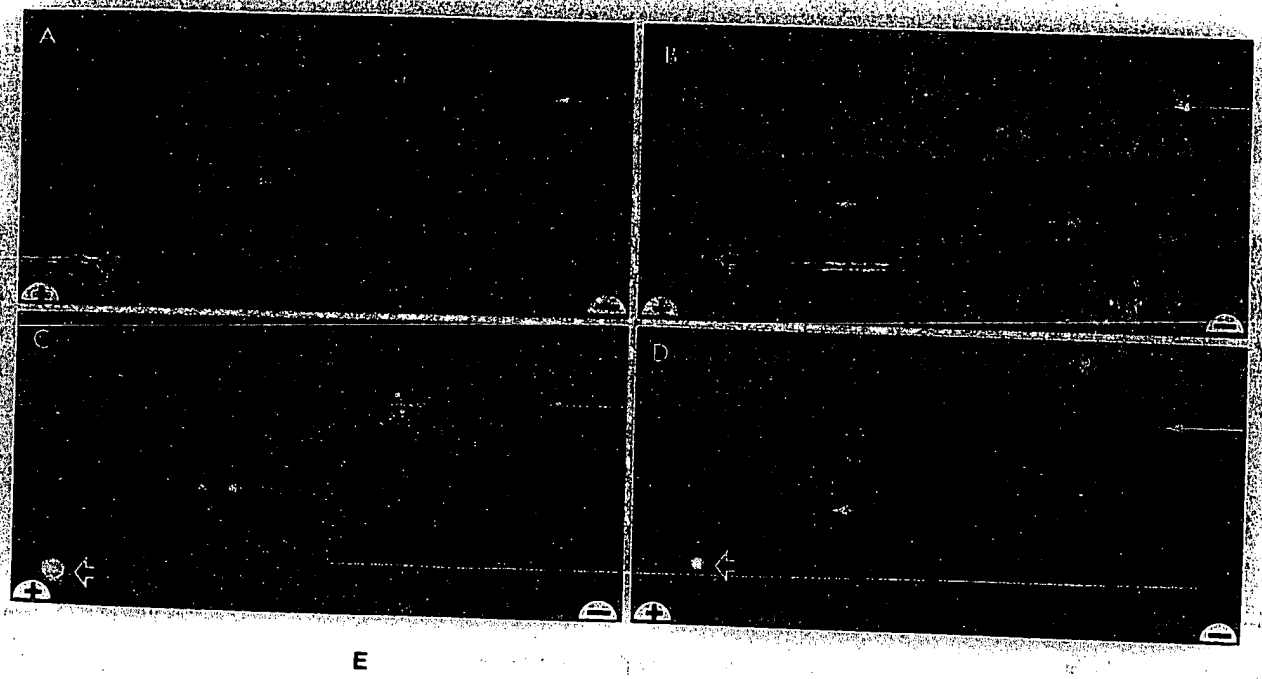
Expression of calponin and SM 22 during development

In the basic region of two-dimensional electrophoresis gels of whole adult muscle homogenates, two proteins, exhibiting multiple isoelectric isoforms, were conspicious (Fig. 1). These corresponded to calponin (34 kDa) and SM 22 (22 kDa). The

number of isoforms observed varied between species, three being detected for both proteins in chicken gizzard (Fig. 1a) and four in pig stomach (Fig. 1b); in guinea pig taenia coli we detected four isoforms (data not shown). Monoclonal and polyclonal antibodies, specific for calponin, or polyclonal-antibody fractions that cross-reacted with both SM 22 and calponin labelled all isoforms of these proteins (Fig. 2). As shown in Fig. 3, calponin, from both avian and porcine tissues, exhibited the same isoform pattern after purification.

Analysis of different developmental stages of chicken gizzard indicated that the expression of calponin and SM 22 begins synchronously around embryonic day 10, but only as one isoform (Fig. 4). Immunoblotting of two-dimensional gels of samples taken at subsequent developmental stages (Fig. 5) showed that the single isoforms of calponin, as well as SM 22, persisted until well after hatching. Only at post hatching day 10 could a second isoform be observed (Fig. 5c); all three isoforms were expressed in the adult (21 weeks; Fig. 5d). For porcine stomach, more or less the same pattern of appearance of isoforms was observed, starting with the two most basic α and β isoforms (Fig. 6).

We have previously shown that calponin expression is down-regulated in chicken gizzard cells cultivated *in vitro* and that this down regulation parallels that of metavinculin and



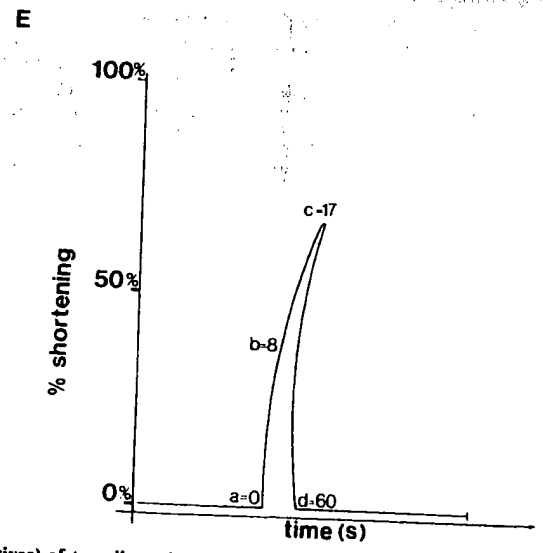


Fig. 8. Autoradiograms (printed as negatives) of two-dimensional gels of phosphate radiolabelled muscle strips. The strips were snap-frozen at steps in the contraction/relaxation cycle corresponding to points A – D in the tension trace shown in (E) (see also Methods). Open arrow, 20-kDa myosin light chain; short arrow, 28-kDa protein of Park and Rasmussen [30]; long arrow, position of calponin as determined from parallel Coomassie blue staining. (E) Typical phasic contraction response of chicken gizzard muscle to 10^{-4} M carbachol indicating time points a – d (times in seconds) at which muscle strips were taken for phosphate-incorporation analysis.

the smooth-muscle-specific isoform of caldesmon [28]. Maintenance of the cells in low serum (2%), to retard proliferation, resulted in continued expression of both calponin and SM 22 for up to one week in primary culture (Fig. 7a). However, growth of cells in 10% serum followed by replating, resulted in a typical modulation of the smooth muscle phenotype [29] that was characterized by the loss of calponin expression in secondary culture (Fig. 7b). It was noteworthy, however, that the expression of SM 22 was not down regulated under these conditions, even after subsequent replatings (Fig. 7b).

Calponin and SM 22 isoforms *in vivo* are not derived via phosphorylation

Winder and Walsh [9a] have recently suggested that the reversible phosphorylation of calponin (observed *in vitro*) is involved in the regulation of the actin-myosin interaction in smooth muscle. To test this idea and to gain more insight into the origin of calponin isoforms, we have analyzed living smooth-muscle strips at different steps in the contraction cycle after loading with radioactive phosphate. We employed muscle strips from chicken gizzard and guinea pig taenia coli, with essentially the same result; those using chicken gizzard are presented here. Fig. 8e shows the tension response of adult chicken gizzard strips in response to 10^{-4} M carbachol. Typically, only brief phasic responses were observed, followed by a return to basal tension in the continued presence of the agonist. Parallel muscle strips loaded with $^{32}PO_4$ and contracted in the same way were snap-frozen at the different stages indicated (Fig. 8a–d) and subjected to two-dimensional-gel autoradiography. The autoradiograms showed significant and expected incorporation into the myosin 20-kDa light chain on contraction (Fig. 8b and c), as well as into the 28-kDa protein, described earlier by Park and Rasmussen [30]

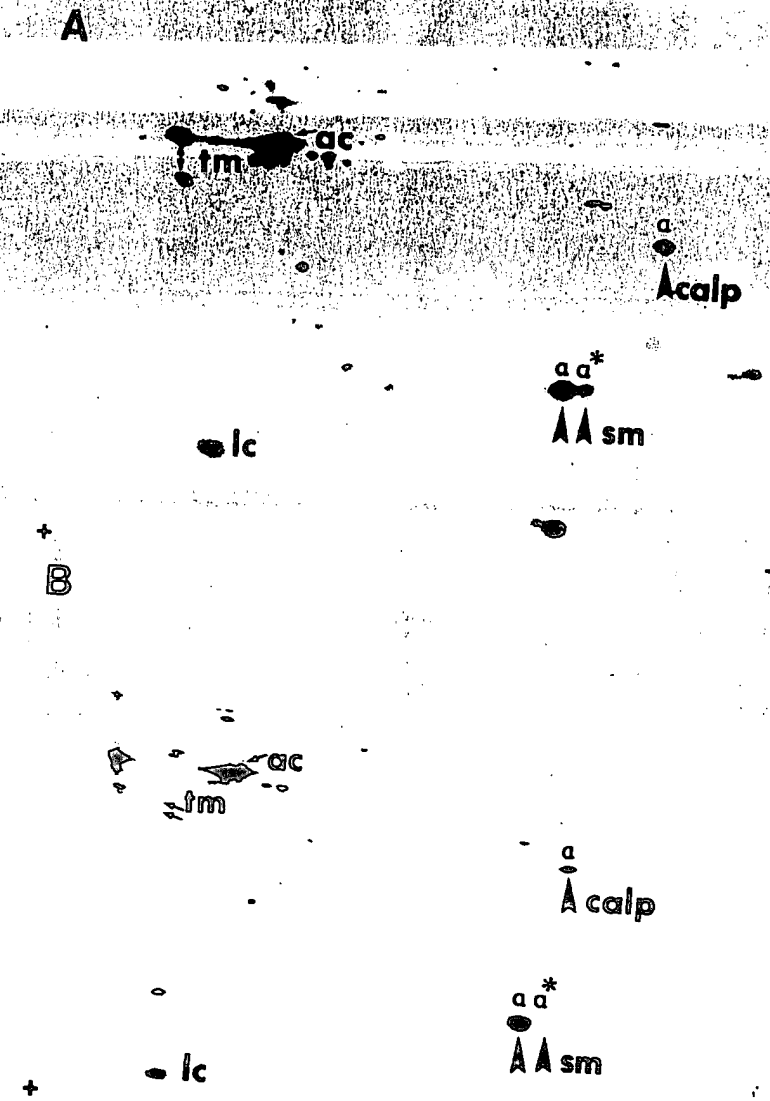


Fig. 9. Autoradiograms of the *in vitro* translation products of total mRNA from (a) chicken gizzard and (b) hog stomach. α*. A polypeptide adjacent to the SM 22 α isoform not observed in the tissue samples (see also text). Tm, tropomyosin; ac, actin; lc, 20-kDa myosin light chain; sm, SM 22.

and Colburn et al. [31]. Under no conditions, however, could any incorporation be detected in either calponin or SM 22.

Immunoblotting of two-dimensional gels of adult muscle, with a monoclonal anti-phosphoserine antibody, showed no reaction with any of the calponin isoforms and the isoform pattern of purified calponin was unaffected by incubation with alkaline and acidic phosphatases or crude phosphatases purified from chicken gizzard smooth muscle (not shown).

Smooth muscle mRNA encodes a single 34 kDa isoform of calponin in an *in vitro* translation system

In vitro translation of mRNA, purified from adult chicken gizzard or pig stomach smooth muscle in either a wheat-germ or reticulocyte translation system, gave rise to only one isoform of calponin (Fig. 9a and b). Coelectrophoresis of the radioactive translation products with unlabelled whole muscle samples (not shown) facilitated the assignment of the *in vitro* translated isoforms as the most basic α variants. For SM 22, two translation products were observed (Fig. 9a and b), one in the position of the α isoform and a second, even more basic polypeptide (α^*) not observed in tissue samples (see Discussion). This assignment of the positions of the translated products was confirmed by immunoblotting parallel gels with calponin/SM-22 cross-reactive antibodies (not shown).

DISCUSSION

In their studies on aorta calponin, Takahashi et al. [32] described the presence of isoelectric isoforms, but these were not well resolved on their one-dimension NEPHGE gels. We show here that avian gizzard and porcine stomach exhibit three and four isoforms, respectively, in the adult. The data obtained with avian gizzard can be directly correlated with *in vitro* functional studies of calponin, since this tissue serves as a common source of smooth muscle contractile proteins. Interest in the modifications underlying the multiple calponin isoforms was intensified by the findings of Winder and Walsh [9a] and Naka et al. [10] that phosphorylation of calponin *in vitro* antagonizes the binding of calponin to actin and releases the inhibitory effect that this protein has on the Mg^{2+}-ATPase of smooth muscle actomyosin. As we show, none of the calponin isoforms were phosphorylated to any detectable level *in vivo* under conditions for which reversible phosphorylation of the myosin light chain could be shown. These data on gizzard confirm the recent results of Barany and colleagues [18] using porcine aorta. The tension responses that we obtained with gizzard strips were only phasic in nature; however, in parallel studies with living guinea pig taenia coli strips that readily undergo tonic contraction, calponin remained likewise unphosphorylated during contraction and relaxation. We conclude, in agreement with Barany et al. [18], that calponin is inaccessible *in vivo* to the kinases found to phosphorylate this protein *in vitro*.

The specific appearance of multiple calponin isoforms late during smooth muscle differentiation suggests that these isoforms may be important for the proper function of the differentiated cell. It is noteworthy that the accumulation of multiple isoforms correlates with the appearance, around one week after hatching, of smooth-muscle-specific tropomyosins [33] (Fig. 4), especially in the context of the demonstrated binding of calponin to tropomyosin [16, 34]. It remains to be shown whether calponin has higher affinities for smooth muscle tropomyosin compared to the non-muscle tropomyosin isoforms expressed during early gizzard development [33]. Unlike the tropomyosin isoforms, those of calponin do not appear to arise via alternative splicing or from multiple genes, since only a single isoform of 34 kDa was evident after *in vitro* translation of total smooth muscle mRNA. In a recent report, Takahashi et al. [17] described cDNA clones encoding calponin and noted the existence of two species of cDNA in chicken. Their second 'β' isoform encoded a protein of lower molecular mass (28 kDa) than the major calponin variant in gizzard, and they suggest that these variants arise via alternative splicing. This lighter isoform might correspond to the low molecular mass calponin that is strongly expressed in the human urogenital tract [35]. We detected a low molecular mass protein in gizzard that cross-reacted with the specific calponin antibody in immunoblots of whole muscle samples, but this variant was expressed in very low amounts.

The expression of calponin is not wholly restricted to smooth muscle, as indicated by the recent demonstration by Takeuchi et al. [33] of calponin in blood platelets. This finding is in line with the synthesis by these cells of other proteins, like metavinculin [34] that are also major smooth muscle products. Takeuchi et al. [33] further claim to have identified a second immunoreactive form of calponin in platelets of molecular mass 22–23 kDa. In view of the molecular similarities shared by calponin and SM 22, their protein could correspond to platelet SM 22.

The isoforms and properties of SM 22 have been described earlier by Lees-Miller et al. [9, 15] and Pearlstone et al. [38]. We add here the change in isoform pattern of SM 22 during development, that mirrors closely that seen with calponin. Although Lees-Miller et al. [9] could find no binding partners for SM 22 in their studies, the subsequent identification of sequence similarities of this protein not only with calponin, but in other regions, with proteins expressed in muscle of *Drosophila* and *Caenorhabditis elegans* [16] makes the search for SM 22 function all the more intriguing. According to Lees-Miller et al. [9] the SM 22 β isoform exhibits a different amino acid composition to the α isoform. We confirm their data that the SM 22 isoforms do not arise via differential phosphorylation. Further, the appearance of two polypeptides in the *in vitro* translation system may suggest the existence of two different mRNA species for this protein, in line with the above data [9]. Further sequence analysis would be required to settle this question.

The most striking result with regard to SM 22 was the retention of the α isoform in subcultures of gizzard smooth muscle after down-regulation of other smooth muscle markers had occurred, suggesting that SM 22 expression in culture may serve as a marker of terminal modulation of the smooth muscle phenotype *in vitro*.

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ANTI-PHOSPHOSERINE AND ANTI-PHOSPHOTHREONINE ANTIBODIES MODULATE AUTOPHOSPHORYLATION OF THE INSULIN RECEPTOR BUT NOT EGF RECEPTOR

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Received September 1, 1993

Summary: We examined the effect of anti-phosphothreonine and anti-phosphoserine antibodies on insulin receptor autophosphorylation. These antibodies did not affect insulin binding activity of the receptor. These antibodies, however, inhibited insulin-stimulated autophosphorylation of insulin receptor, while did not affect EGF-stimulated autophosphorylation of EGF receptor. The inhibition was reversed by adding large amounts of phosphoserine or phosphothreonine. These data suggest that phosphoserine and phosphothreonine on insulin receptor play an important role in insulin-induced conformational change of the receptor.

Insulin receptor is a heterotetrameric glycoprotein consisting of two α- and two β-subunits. Insulin binds to the α-subunit which is external to the cell membrane. This interaction induces the conformational change of the receptor and stimulates a tyrosine kinase activity present at the cytoplasmic portion of the β-subunit, leading to autophosphorylation of the receptor on tyrosine residues in intact cells and in cell-free preparations of the receptor(1,2).

A number of polyclonal and monoclonal antibodies against the insulin receptor as well as anti-phosphotyrosine antibodies(αP-Tyr) have been shown to be useful to study the structure-function relationship of the receptor(3-8). It has been demonstrated that anti-phosphotyrosine antibodies modulate (increase or decrease) insulin receptor kinase activity, probably through affecting a conformational change of the receptor(4-6).

Serine and threonine residues of the β-subunit are phosphorylated at the basal state and enhanced when intact cells are stimulated with insulin(2,9). It has been reported that the phosphorylation on serine and threonine residues may modulate the receptor kinase activity through unknown mechanisms(10). Recently we have produced anti-phosphoserine(αP-Ser) and anti-phosphothreonine(αP-Thr) antibodies which are able to recognize various proteins phosphorylated on serine and threonine residues including insulin and EGF receptors(11).

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In the present study we examined whether the anti-phosphoserine and anti-phosphothreonine antibodies modulate the insulin receptor function.

Materials & Methods

Cells and cell culture

CHO-HIR cells which express a large amount of human insulin receptors in Chinese hamster ovary cells were prepared by using vectors containing the mouse dihydrofolate reductase gene as described previously(12). CHO-HIR cells were harvested with 1 μM methotrexate in α-minimum essential medium(αMEM)(without deoxynucleosides) supplemented with 10% dialyzed fetal bovine serum. Human epidermoid carcinoma A431 cells were grown in Dulbecco's modified Eagle's medium.

Preparation of antibodies toward phosphoserine(P-Ser) and phosphothreonine(P-Thr)

Antibodies toward P-Ser and P-Thr(αP-Ser and αP-Thr)were prepared as described previously(11).

Effects of αP-Ser and αP-Thr on insulin receptor autophosphorylation

Insulin receptor was partially purified from rat liver and CHO-HIR cells on a wheat germ agglutinin(WGA) column by the methods as described previously(13). Briefly, CHO-HIR cells and rat liver were homogenized in 50 mM Hepes(pH 7.4), 1% Triton X-100, 5 mM EDTA, 2mM phenylmethylsulfonyl fluoride(PMSF) and 0.1mg/ml aprotinin (buffer A) at 4°C and centrifuged at 150,000g for 60min. The supernatant was applied to WGA affinity column and the column was eluted with 0.3M N-acetyl glucosamine in buffer B(50mM Hepes(pH 7.4), 0.1% TritonX-100, 2mM PMSF, 0.1mg/ml aprotinin). The insulin receptor was further purified with insulin-agarose by the methods of Lewis et al(9,14).
Partially purified insulin receptor was incubated with various concentrations of αP-Ser, αP-Thr or αP-Tyr(15) at 22°C for 2h. After insulin(final concentration,10^{-7}M) was added at 22°C for 30min, the mixture was phosphorylated for 5min in 50mM Hepes(pH 7.4),100μM[γ-^{32}P]ATP, 5mM $MnCl_2$, and 0.1% Triton X-100. The reaction was terminated by boiling in Laemmli's sample buffer. The mixture was then subjected to 7.5% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis(SDS-PAGE), followed by autoradiography.

Effect of αP-Ser and αP-Thr on EGF receptor autophosphorylation

EGF receptor was partially purified from A431 cells with WGA column. A431 cells were homogenized in buffer A and centrifuged at 150,000g for 60 min. The supernatant was applied to WGA column and the column was eluted with 0.3M N-acetyl glucosamine in buffer B. The effect of αP-Ser and αP-Thr on EGF receptor autophosphorylation was examined as described above.

Results & Discussion

Effect of αP-Ser and αP-Thr on insulin receptor autophosphorylation

αP-Ser and αP-Thr were studied to see if they affect the insulin receptor autophosphorylation. After incubation of the WGA-purified insulin receptor from CHO-HIR cells with 10μg/ml of αP-Ser, αP-Thr or αP-Tyr, insulin was added to autophosphorylate the receptor. As shown in Fig.1, both αP-Ser and αP-Tyr considerably inhibited the autophosphorylation of the receptor, while αP-Thr partially inhibited at the protein concentration of 10μg/ml. Insulin-stimulated phosphorylation of 220-kDa and other minor proteins was also inhibited by these antibodies. In order to examine whether the inhibition of the autophosphorylation is due to a direct binding of

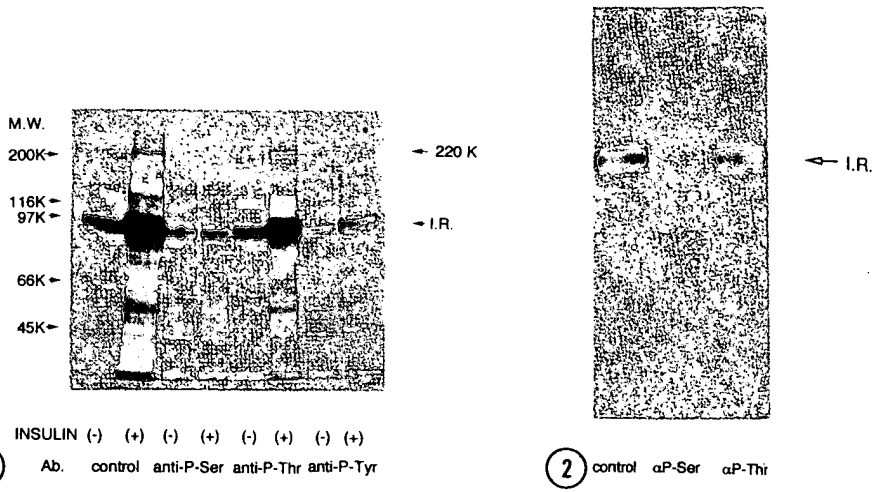


Fig.1. Inhibition of WGA-purified insulin receptor autophosphorylation by αP-Ser,αP-Thr and αP-Tyr; WGA-purified insulin receptor from CHO-HIR cells was incubated with 10 μg/ml of control IgG, αP-Ser, αP-Thr or αP-Tyr, stimulated with 10^{-7}M insulin and then phosphorylated by adding [γ-^{32}P]ATP. Phosphorylated insulin receptor (95-kDa) was separated on 7.5% SDS-PAGE and visualized by autoradiography.

Fig.2. Inhibition of insulin-agarose-purified insulin receptor autophosphorylation by αP-Ser and αP-Thr; WGA-purified insulin receptor was further purified with insulin-agarose.The effect of 10 μg/ml of control IgG, αP-Ser or αP-Thr on highly-purified insulin receptor autophosphorylation was examined as described in Fig.1 and "Materials & Methods".

the antibodies to the receptor, WGA-purified insulin receptor was further purified with insulin-agarose. The autophosphorylation of the highly-purified insulin receptor was also inhibited completely by αP-Ser and partially by αP-Thr at the protein concentration of 10μg/ml (Fig.2). Dose dependency of the inhibition was next examined in Fig.3. Ten μg/ml of αP-Ser inhibited insulin-stimulated autophosphorylation of the receptor to the

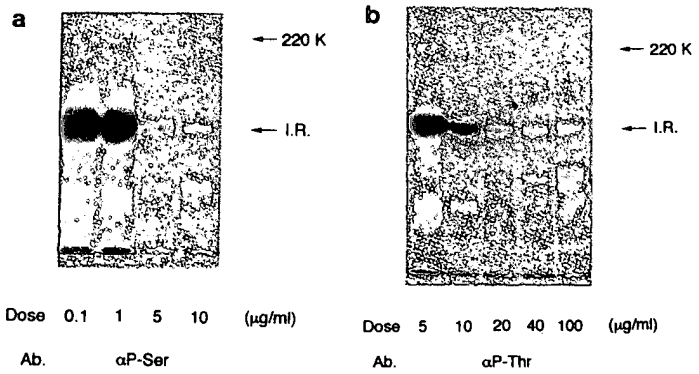


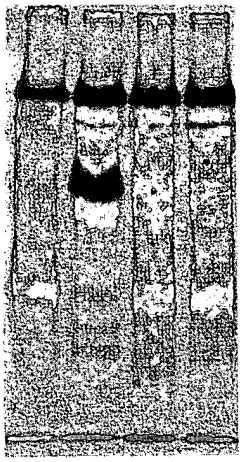
Fig.3. Inhibition of insulin receptor autophosphorylation by various concentrations of αP-Ser and αP-Thr; WGA-purified insulin receptor was incubated with the indicated concentrations of αP-Ser(a) and αP-Thr(b). Insulin receptor autophosphorylation was examined as described in "Materials & Methods".

basal level (before insulin stimulation) while a protein concentration of more than 20μg/ml was necessary to give rise to this degree of the inhibition in the case of αP-Thr. Insulin-stimulated phosphorylation of 220-kDa protein was also affected with a similar dose-dependency. Both antibodies did not affect insulin binding activity of the receptor(data not shown).

Effect of αP-Thr and αP-Ser on EGF receptor autophosphorylation

WGA eluate from rat liver, which contains both insulin and EGF receptors, was incubated with 20 μg/ml of αP-Thr. Insulin was added and then the proteins were phosphorylated. Phosphoproteins were analyzed by SDS-PAGE and autoradiography(Fig.4). αP-Thr completely inhibited insulin receptor autophosphorylation. In contrast, αP-Thr did not affect the phosphorylation of EGF receptor which is known to be partially activated *in vitro* after cell solubilization.

EGF receptor was purified from human epidermoid carcinoma cell(A431 cells) with the WGA column. After incubation with 20 μg/ml of αP-Thr, the EGF receptor was stimulated by EGF and phosphorylated(Fig.5). Autophosphorylation of the receptor was not affected by αP-Thr. Even at a higher concentration of αP-Thr, both the binding



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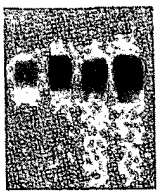

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Fig.4. Effect of αP-Thr on phosphorylation of the insulin and EGF receptor; Insulin and EGF receptor were purified from rat liver with WGA column. They were preincubated with 20 μg/ml of control IgG or αP-Thr, stimulated with 10^{-7}M insulin, and then phosphorylated by adding [γ-^{32}P]ATP. Phosphorylated insulin receptor (95-kDa) and EGF receptor (170-kDa) were separated on 7.5% SDS-PAGE and visualized by autoradiography.

Fig.5. Effect of αP-Thr on EGF receptor autophosphorylation; EGF receptor was purified from A431 cells with WGA column. EGF receptor was preincubated with 20 μg/ml of control IgG or anti-P-Thr, stimulated by 200 ng/ml EGF, and then phosphorylated by adding [γ-^{32}P]ATP. Phosphorylated EGF receptor was separated on 7.5% SDS-PAGE, followed by autoradiography.

activity and autophosphorylation of EGF receptor were not inhibited(data not shown). αP-Ser did not cause any significant changes in EGF receptor autophosphorylation either(data not shown). Furthermore, EGF-induced autophosphorylation of EGF receptor in the membrane preparation was not inhibited by these antibodies(data not shown).

Reversibility of the effect of αP-Thr and αP-Ser on insulin receptor autophosphorylation

To see whether or not the inhibition is reversible, 20 mM of P-Thr was added to the WGA-purified insulin receptor preincubated with 10 μg/ml of αP-Thr. The receptor was then further incubated with insulin, and phosphorylated(Fig.6). Inhibition of the autophosphorylation was reversed by a large amount of P-Thr. However, phosphorylation of EGF receptor was not affected by these procedures. Inhibition of insulin receptor autophosphorylation by αP-Ser was also reversible(data not shown). These results suggest that specific binding of the antibody to P-Thr or P-Ser on insulin receptor results in the inhibition of insulin-induced autophosphorylation of the receptor.

αP-Tyr has also been shown to decrease or increase the insulin receptor kinase activity(4-6). Although the reason for such contradictory results is unknown, it has been proposed that αP-Tyr locks the conformation of the insulin receptor in tyrosine kinase active or inactive form(6). In the present study, we examined whether αP-Ser and αP-

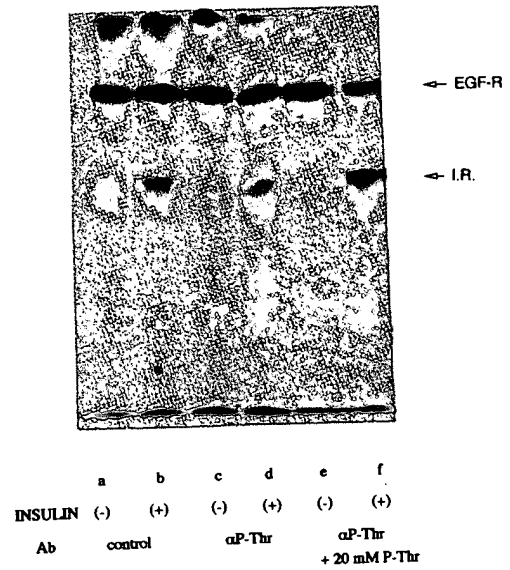

Fig.6. Reversal of the antibody-induced inhibition of WGA-purified insulin receptor autophosphorylation; WGA-purified insulin receptor from rat liver was incubated with 10 μg/ml of control IgG (lanes a and b) or αP-Thr (lanes c, d,e and f). The receptors were then stimulated with (lanes b,d and f) or without 10^{-7}M insulin (lanes a,c and e) and phosphorylated by adding [γ-^{32}P]ATP. In lanes e and f, 20 mM of P-Thr was added before the insulin stimulation. Phosphorylated insulin receptor (95-kDa) was separated on 7.5% SDS-PAGE, followed by autoradiography.

Thr also modulate the insulin and EGF receptor function. We found that the antibodies inhibited autophosphorylation of the insulin receptor but not EGF receptor. When the antibodies and [γ-^{32}P]ATP were incubated simultaneously with the receptor for 5 min, receptor autophosphorylation was not inhibited at all. Thus it is unlikely that these antibodies bind [γ-^{32}P]ATP in a non-specific manner, making ATP less available for the reaction. It seems also unlikely that these antibodies activated phosphotyrosine phosphatase activities present in the WGA eluate, since the effect of the antibodies was also seen using insulin-agarose-purified insulin receptor in the presence of phosphatase inhibitors(data not shown). Insulin receptor is phosphorylated on a number of different serine residues(16), as well as threonine residues(2,9)without insulin stimulation in intact cells. Serines 1293 and 1294 and threonine 1336 have been proposed as sites of insulin- and phorbol ester-stimulated insulin receptor phosphorylation(9,17). It was recently reported that serine in the juxtamembrane region of the insulin receptor was phosphorylated before insulin stimulation and insulin enhanced its phosphorylation(18). Although the antibody recognition sites are not known, our data favor the possibility that these antibodies react with some phosphothreonine and phosphoserine residues on the insulin receptor before insulin stimulation and lock the receptor into the kinase inactive form. Our data suggest that the phosphorylation on serine and threonine residues of the insulin receptor play an important role in inducing conformational change of the receptor upon insulin binding.

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Neuroscience Letters

Autoradiographic localization of the [^{3}H]-(*S*)-zacopride labelled 5-HT$_3$ receptor in porcine brain

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Abstract

Using the technique of in vitro receptor autoradiography, we have determined the distribution of the [^{3}H]-(*S*)-zacopride labelled 5-HT$_3$ receptor in porcine brain. Highest densities of 5-HT$_3$ receptor-associated [^{3}H]-(*S*)-zacopride binding were detected in areas of porcine spinal cord, nodose ganglion, trigeminal nerve nucleus, area postrema and cerebral cortex, with relatively lower levels in other brain regions (e.g. hippocampus, caudate-putamen). The distribution of [^{3}H]-(*S*)-zacopride binding in porcine forebrain provides further evidence for inter-species differences with respect to the differential expression of the 5-HT$_3$ receptor in the forebrain.

Keywords: 5-HT$_3$ receptor; [^{3}H]-(*S*)-zacopride; Autoradiography; Porcine brain

The autoradiographic distribution of the central 5-HT$_3$ receptor has been characterized in several species using a variety of radioligands. We presently report the localization of the 5-HT$_3$ receptor in porcine brain, investigated using the selective 5-HT$_3$ receptor radioligand [^{3}H]-(*S*)-zacopride [2]. We have previously used this radioligand to pharmacologically characterize the 5-HT$_3$ receptor expressed in pig cerebral cortex [5]. The distribution of the 5-HT$_3$ receptor in pig brain is likely to be of particular interest given the known inter-species variations with respect to the central distribution of the 5-HT$_3$ receptor (for review see Ref. [3]), and the recent identification of both 5-HT$_{3A}$ and non-5-HT$_{3A}$ proteins in the 5-HT$_3$ receptor complex purified from pig brain [6]. Preliminary results have been communicated to the British Pharmacological Society [4].

Pig brain tissue was obtained from a local abattoir within 30 min of death and transported over ice. Tissues were dissected under cold room conditions, using a scalpel to isolate regions of interest, and frozen at −80°C within 2 h of death. Piglet brain regions were immediately frozen in liquid nitrogen following removal from anaesthetized (halothane then maintained on Saffan) piglets (mixed sex; up to 1 week old), and stored at −80°C. Brain sections (20 μm) were cut using a cryostat (−14 to −21°C), thaw mounted on to gelatin-coated glass slides, and stored at −80°C until use. Thawed (4°C) slide-mounted sections were pre-incubated in Tris/Krebs buffer (mM: Tris, 50.0; NaCl, 118.0; KCl, 4.75; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; glucose, 11.0; pH 7.4) for 30 min at 4°C, before being incubated in Tris/Krebs buffer (4°C) containing 0.4 nM [^{3}H]-(*S*)-zacopride (78 Ci/mmol, Amersham) in the absence (total binding) or presence (non-specific binding) of granisetron (1 μM) for 60 min. Subsequently, the sections were washed twice for 5 min in ice-cold Tris/Krebs buffer, and rinsed for 1 s in ice-cold distilled water. The sections were then rapidly dried in a stream of cold dry air before being exposed to tritium-sensitive film (Hyperfilm-[^{3}H], Amersham) along with tritium standards (fmol/mg grey matter tissue equivalent; Amersham) for 14 weeks. Developed autoradiographs were analyzed and quantified (with reference to the tritium standards) using image analysis (MCID, Imaging Research). Total and non-specific binding was determined for each area from 6–53 sections per animal, originating from one to six separate animals.

Specific binding (non-specific binding defined by the presence of granisetron (1 μM), which represented between approximately 90–20% of total binding) of [^{3}H]-(*S*)-zacopride (0.4 nM) was differentially distributed throughout the forebrain of the pig (Table 1 and Fig. 1). In contrast, non-specific binding was distributed homogeneously. High levels of specific [^{3}H]-(*S*)-zacopride binding were detected in pig and piglet cerebral cortex, with the binding being largely associated with the outer layers of the cerebral

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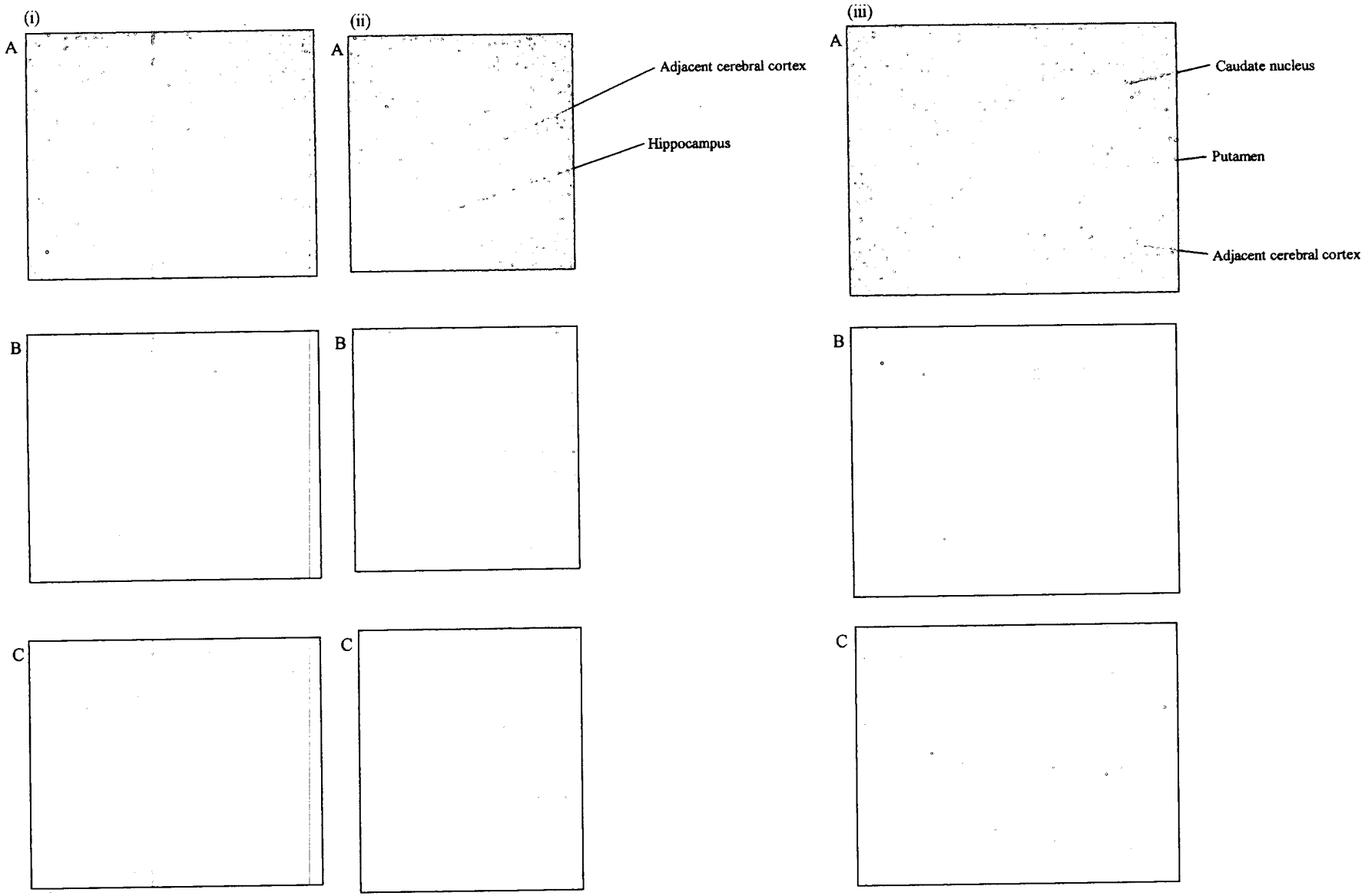

Fig. 1. Representative cresyl-violet stained sections of the pig forebrain (A), with the corresponding autoradiographs obtained using 0.4 nM [^{3}H]-(*S*)-zacopride in the absence (total binding; (B)) and presence (non-specific binding; (C)) of granisetron (1 μM). Examples from three regions are shown, which include (i) frontal cortex; (ii) hippocampus, and (iii) caudate-putamen.

Table 1
Regional distribution of specific [^{3}H]-(*S*)-zacopride (0.4 nM; non-specific binding defined by granisetron, 1 μM) binding in pig central nervous system and piglet nodose ganglion, determined by in vitro autoradiography[a]

Pig brain region	Specific binding (fmol/mg tissue)	Range	*n*
Spinal cord (substantia gelatinosa)	11.38	N/A	1
Piglet nodose ganglion	10.04	N/A	1
Trigeminal nerve nucleus	9.35	N/A	1
Frontal cortex	4.75	3.28–5.59	3
Striate cortex	3.77	N/A	1
Area postrema	3.31	N/A	1
Parietal cortex	3.06	2.04–4.07	2
Temporal cortex	2.31	1.15–3.32	5
Occipital cortex	2.27	1.80–2.73	2
Globus pallidus	0.91	N/A	1
Hippocampus	0.79	0.31–1.75	6
Caudate-putamen	0.49	0.01–0.78	6
Cerebellum	0.41	N/A	1
Amygdala	0.24	N/A	1

[a] Total and non-specific binding was determined for each area from 6–53 sections per animal, originating from *n* separate animals.

cortex (Fig. 1). Lower levels of specific [^{3}H]-(*S*)-zacopride binding were detected in various other brain regions (globus pallidus, hippocampus, caudate-putamen, cerebellum and amygdala, see Table 1).

Highest levels of specific [^{3}H]-(*S*)-zacopride binding were detected in porcine spinal cord (substantia gelatinosa), nodose ganglion, trigeminal nerve nucleus and area postrema, however, due to the difficulty in obtaining hindbrain regions during brain removal, only limited data was obtained for these regions which therefore cannot be included in a quantitative analysis.

In all species investigated to date the highest concentrations of central 5-HT$_3$ receptors are found in the hindbrain, within a subregion of the nucleus tractus solitarius (the subnucleus gelatinosus), and lower levels in the remainder of the nucleus tractus solitarius, area postrema, dorsal motor nucleus of the vagus nerve and the trigeminal nerve nucleus [9]. In the forebrain, the relative distribution of 5-HT$_3$ receptors varies between species. For instance, in rodent forebrain, 5-HT$_3$ receptor expression is relatively high in limbic brain regions (e.g. hippocampus, cerebral cortex, amygdala) whilst levels are below or near the limit of detection in extrapyramidal brain regions (e.g. striatum (caudate-putamen), see Ref. [8] and references therein). This contrasts markedly with the distribution of 5-HT$_3$ receptors in the human forebrain, where levels in the cerebral cortex are relatively low (although relatively high in the amygdala and hippocampus), whilst levels are relatively high in human extrapyramidal brain regions (caudate nucleus and putamen, see Ref. [8] and references therein).

In common with other species, the present studies suggest that the highest levels of specific [^{3}H]-(*S*)-zacopride binding are located in porcine spinal cord, nodose ganglion, trigeminal nerve nucleus and area postrema. The high density of 5-HT$_3$ receptors in the nodose ganglion and area postrema probably reflects the role of this receptor in the control of the emetic response, as has been proposed in other species [1]. Other regions of the hindbrain (e.g. nucleus tractus solitarius) were not able to be examined due to damage to these regions during brain removal at the abattoir.

The distribution of specific [^{3}H]-(*S*)-zacopride binding in the pig forebrain provides further evidence of inter-species differences with respect to the 5-HT$_3$ receptor, and confirms our earlier study examining the distribution of 5-HT$_3$ receptors in homogenates of pig brain [5].

The presence of 5-HT$_3$ receptors in cortical and hippocampal regions of the brain may reflect the proposed involvement of this receptor in cognitive processes (see Ref. [3] for review). The 5-HT$_3$ receptor has also been implicated in anxiety. Hence, the expression of 5-HT$_3$ receptors within the amygdala, in most species examined, may provide the site of the anxiolytic action of 5-HT$_3$ antagonists [7]. The significance of 5-HT$_3$ receptor recognition sites in limbic areas of the brain may be related to the use of antagonists at this receptor site as putative psychotropic agents, and to reduce the rewarding properties and withdrawal symptoms associated with drugs of abuse (see Ref. [3] for review).

In conclusion, the present studies have identified high densities of the [^{3}H]-(*S*)-zacopride labelled 5-HT$_3$ receptor in areas of porcine cerebral cortex, and provide further evidence for inter-species differences with respect to the 5-HT$_3$ receptor. This confirms and extends our previous studies using homogenates of pig brain [5]. Further studies are needed to determine the relative distribution of the 5-HT$_{3A}$ and non-5-HT$_{3A}$ proteins associated with the 5-HT$_3$ receptor complex in porcine brain.

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PREPARATION OF ANTI-PHOSPHOSERINE AND ANTI-PHOSPHOTHREONINE ANTIBODIES AND THEIR APPLICATION IN THE STUDY OF INSULIN- AND EGF- INDUCED PHOSPHORYLATION

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Summary: We prepared antibodies against phosphoserine (P-Ser) and phosphothreonine (P-Thr) by immunizing rabbits with P-Ser or P-Thr conjugated to bovine serum albumin. The antibodies (anti-P-Ser and anti-P-Thr) were purified using P-Ser or P-Thr affinity columns. Anti-P-Thr was highly specific for P-Thr, while anti-P-Ser showed weak cross-reacitivity with P-Thr. We showed that these antibodies can immunodetect serine/threonine phosphorylated insulin and epidermal growth factor (EGF) receptors and several proteins which are phosphorylated on serine/threonine residues in response to insulin or EGF stimulation. The antibodies will certainly provide a good tool for discovering novel kinases and substrates involved in signal transduction.

The interaction of insulin with its specific receptors induces a series of events that lead to signal transduction to its intracellular effectors. Insulin stimulates a tyrosine kinase activity of its receptor (1,2). Insulin also enhances phosphorylation on serine and threonine residues of intracellular molecules far more predominantly than on tyrosine residues, which may play important roles in the signal transduction (3-8). However, the total picture of insulin-stimulated serine/threonine phosphorylation remains to be elucidated. In the present study, we attempted to prepare anti-phosphoserine and anti-phosphothreonine antibodies with a high specificity and affinity in order to study insulin-induced serine, threonine phosphorylation in cells.

Materials & Methods

Cells and cell culture

CHO-HIR cells which express a large amount of human insulin receptors in Chinese hamster ovary cells were prepared by using vectors containing the mouse dihydrofolate reductase gene as described previously(9). CHO-HIR cells were harvested with 1mM methotrexate in α-minimum essential medium(αMEM)(without deoxynucleosides)supplemented with 10% dialyzed fetal bovine serum.

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Preparation of antibodies toward phosphoserine (P-Ser) and phosphothreonine (P-Thr)

P-Ser or P-Thr (20mg each, purchased from Sigma) was coupled to 20mg of bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) with 80mg of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide in 10ml reaction mixture (pH 8.5) at 4°C overnight. The conjugate was dialyzed against phosphate-buffered saline (PBS), pH 7.5. Rabbits were immunized with 1mg of P-Ser- or P-Thr-BSA conjugate. Booster injections of the same amount of the conjugate were given at 3, 5, and 7 weeks after the first injection. Serum obtained from rabbits immunized with P-Ser-BSA was passed through a column of CN-Br Sepharose 4B coupled with P-Ser. After extensive washing with PBS, the column was first eluted with 40mM P-Thr (αP-Ser/P-Thr), washed, and then eluted with 40mM P-Ser (αP-Ser). Serum from P-Thr-BSA immunized rabbits was passed through the P-Thr column. Antibodies were collected with 40mM P-Ser (αP-Thr/P-Ser), and then with P-Thr (αP-Thr). Each antibody was dialyzed extensively against PBS.

Immunoprecipitation of phosphoprotein in CHO-HIR cells

Subconfluent CHO-HIR cells were harvested in phosphate -free αMEM for 12h, then [^{32}P]-orthophosphate was added. After 2h, the cells were incubated in the presence or absence of 10^{-7}M insulin for 5min. They were frozen in liquid nitrogen, homogenized in buffer A (50mM Hepes, 20mM NaF, 10mM sodium pyrophosphate, 2mM $NaVO_3$, 5mM EDTA, 2mM phenylmethylsulfonyl fluoride and 0.1mg/ml aprotinin, pH 7.4) including 1.5% Triton X-100 and centrifuged at 150,000g for 60min. The supernatant was applied to wheat-germ-agglutinin (WGA) affinity column and the column was eluted with 0.3M N-acetyl glucosamine in buffer A including 0.1% Triton X-100. The WGA eluate was incubated overnight with αP-Thr, αP-Ser, or αP-Tyr, and then with protein A -Sepharose for an additional 2h at 4°C. The immunoabsorbed phosphoprotein was washed three times with buffer A including 0.1% Triton X-100, boiled in Laemmli's sample buffer, and subjected to 7.5% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblotting of insulin-stimulated phosphoprotein

CHO-HIR cells, stimulated with or without insulin (10^{-7}M), were homogenized and processed as described above without using [^{32}P]-orthophosphate. Fifty μg of solubilized protein was boiled in Laemmli's sample buffer, run on SDS-PAGE and electric-transferred to nitrocellulose membranes. Phosphoproteins on the membranes were immunodetected with αP-Ser, αP-Thr or αP-Tyr(17) as described above.

Stimulation of A431 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA)

A431 cells were washed three times with Dulbecco's PBS and incubated with or without 10^{-8}M TPA for the indicated time. After incubation the cells were solubilized in Laemmli's sample buffer. The phosphoproteins were detected as described above.

Results & Discussion

Characterization of antibodies

The specificity of the antibodies was examined by immunoblotting using P-Ser, P-Thr and P-Tyr coupled to BSA or KLH as antigens (Fig 1). αP-Thr/P-Ser and αP-Thr reacted mainly with P-Thr/BSA. αP-Thr/P-Ser cross-reacted weakly with P-Ser/BSA, when the quantity of antigen was more than 100μg (data not shown). αP-Ser and αP-Ser/P-Thr recognized P-Ser/BSA and weakly P-Thr/BSA. The cross-reactivity with P-Thr/BSA was greater in αP-Ser/P-Thr. These four antibodies cross-reacted very weakly with P-Tyr/KLH, when the quantity of P-Tyr/KLH was more than 10μg. None of the antibodies reacted with BSA (data not shown). Binding of αP-Thr/P-Ser to P-Thr/BSA conjugate, dot-blotted on the nitrocellulose paper, was inhibited by 20mM of both P-Thr and P-Ser but not by 20mM of P-Tyr, Thr or Ser, while that of αP-Thr was inhibited only by P-Thr (data not shown). Binding of αP-Ser to P-Ser/BSA conjugate was also inhibited by 20mM of P-Thr and P-Ser but not by 20mM of P-Tyr, Ser or Thr. These

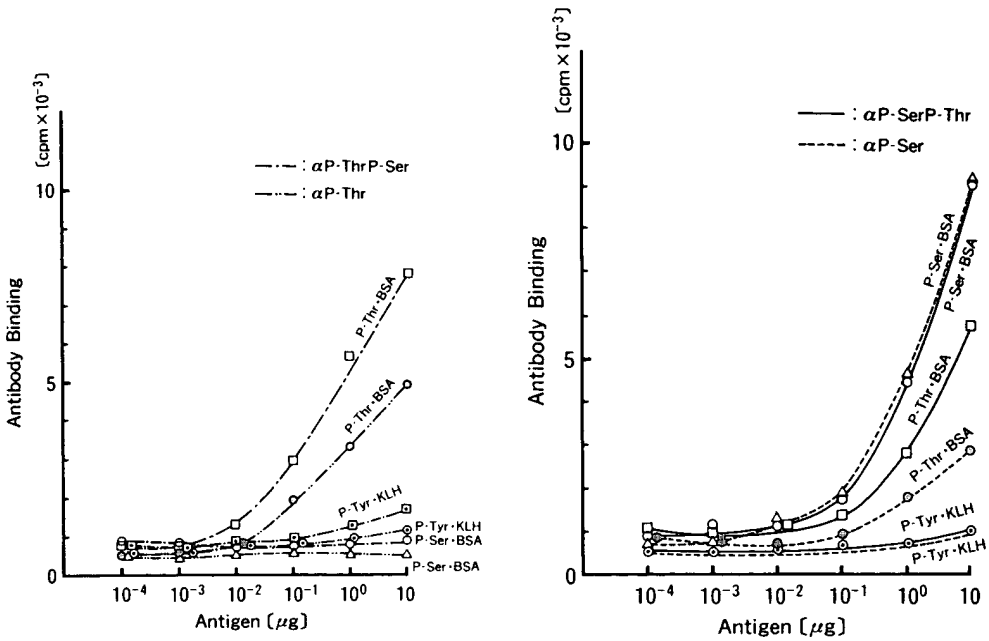

Fig.1. Sensitivity of the antibodies: Various doses of antigens (P-Ser/BSA, P-Thr/BSA and P-Tyr/KLH) on the membrane were reacted with each antibody(5μg/ml) and then [^{125}I]-protein A.

results indicated that the antibodies are produced against phosphoamino acids (P-Ser and P-Thr), and that αP-Thr is highly specific for P-Thr.

Immunodetection of insulin-induced phosphoproteins in the CHO-HIR cells

We checked if these antibodies can immunodetect phosphoproteins, especially insulin receptor, in CHO-HIR cells by both immunoblotting and immunoprecipitation. In the WGA eluate from [^{32}P]-labelled CHO-HIR cells, both αP-Ser and αP-Thr immunoprecipitated insulin receptor(95-kDa phosphoprotein)(Fig 2a). Since the cross-reactivity of these antibodies with P-Tyr is very low, these results indicate that the antibodies detect P-Ser and/or P-Thr on the receptors. αP-Thr immunoprecipitated various phosphoproteins, which were not recognized by αP-Tyr and not stimulated by insulin. As shown in Fig 2b, in the crude Triton X-100 solubilized proteins, αP-Ser immunoblotted 170-kDa phosphoprotein and insulin receptor, which were both stimulated by insulin, while αP-Thr did mainly insulin receptor. On the other hand, αP-Tyr recognized several insulin-induced phosphoproteins including insulin receptor and 185-kDa phosphoprotein (IRS-1) (4,16). Phosphoamino acid analysis of [^{32}P]-labelled insulin receptor revealed that it was mainly phosphorylated on serine residues at the basal condition, while on serine, threonine, and tyrosine residues after insulin stimulation (data not shown). But αP-Ser barely immunodetected insulin receptor at the basal condition. Moreover, this αP-Ser detected fewer phosphoproteins than αP-Thr by both immunoprecipitation and immunoblotting. Differnces in the affinity of the antibody as

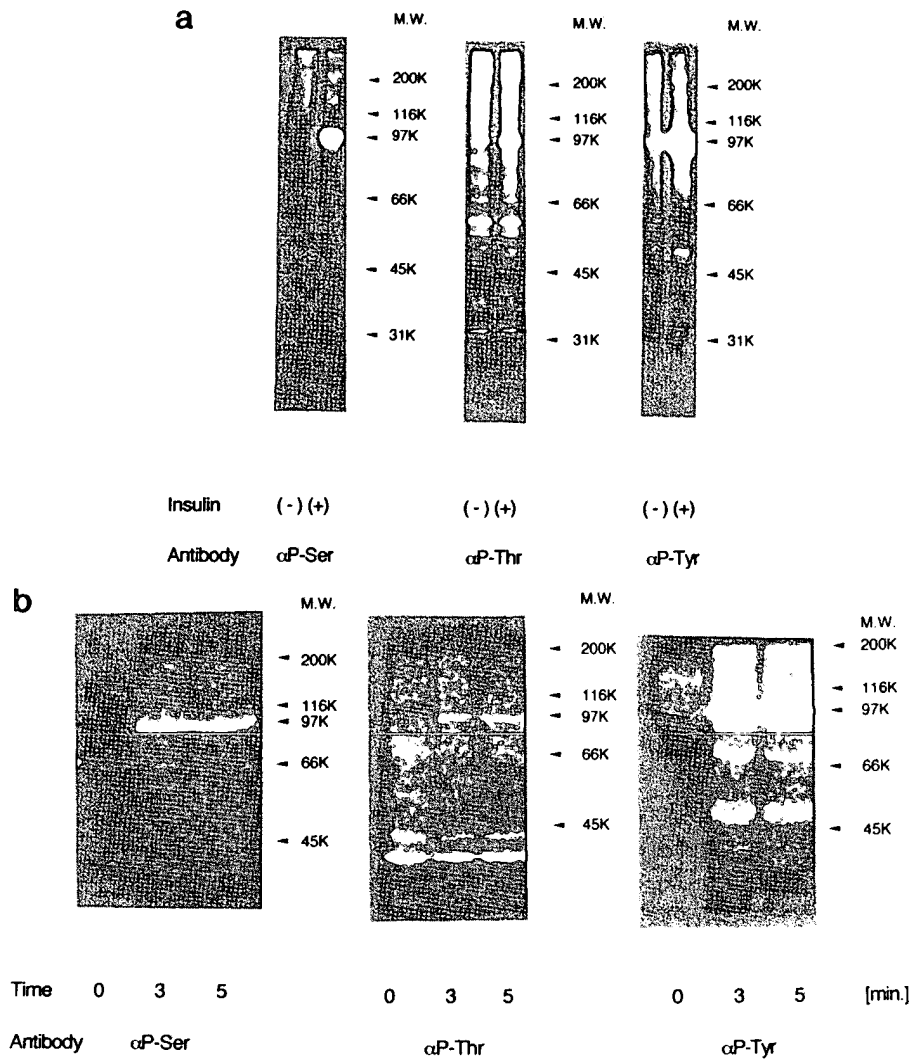

Fig.2 a) Immunoprecipitation of WGA-purified phosphoproteins in [^{32}P]-labelled CHO-HIR cells;CHO-HIR cells, labelled with [^{32}P]-orthophosphate, were stimulated with or without 10^{-7}M insulin for 5 min. WGA-purified protein from the cells was immunoprecipitated with αP-Ser, αP-Thr, or αP-Tyr and subjected to SDS-PAGE.
b) Immunoblotting of insulin-stimulated phosphoproteins in CHO-HIR cells;Triton X-100 solubilized proteins (50μg) from insulin-stimulated CHO-HIR cells were run on SDS-PAGE and immunoblotted with αP-Ser, αP-Thr, or αP-Tyr.

well as the accessibility of the target residues on the molecule by the antibody may account for the findings.

Immunodetection of EGF-stimulated phosphoproteins in A431 cells

Next we examined if these antibodies recognized EGF receptor. The A431 cells were stimulated with EGF for 5 min, solubilized in Triton X-100. The phosphorylated EGF receptor (170 kDa) was immunodetected with αP-Thr and αP-Ser(Fig.3). αP-Thr recognized EGF-stimulated 240-kDa phosphoprotein.

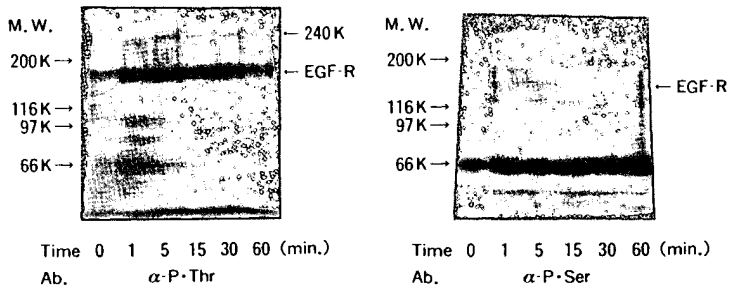

Fig.3. Immunoblotting of EGF-stimulated phosphoproteins in A431 cells:A431 cells were stimulated with 200ng/ml EGF for the indicated time and boiled in Laemmli's sample buffer. The protein was run on SDS-PAGE and immunoblotted with αP-Thr or αP-Ser.

Effect of treatment of A431 cells with phorbol ester

Tumor-promoting phorbol ester, TPA, is reported to activate protein kinase C, which results in phosphorylation of threonine 654 of the EGF receptor and thus inhibits its tyrosine kinase activity (10-13). We investigated if these events could be demonstrated by immunoblotting with αP-Tyr and αP-Thr (Fig.4). Treatment of the cells with TPA resulted in an increase in phosphothreonine (occurring between 1 and 15 min)(Fig.4a) Pretreatment with TPA (15 min) caused dramatic decreases of EGF-stimulated tyrosine phosphorylation of the receptor, 100- and 85-kDa proteins, while it produced increases of EGF-stimulated threonine phosphorylation of the 240- and 40kDa proteins (Fig.4b). Pretreatment with TPA did not change appreciably the phosphothreonine content of the receptor. This may reflect that EGF receptor tyrosine kinase-dependent threonine kinase, which phosphorylates threonine residues on the receptor, is partialy suppressed by protein kinase C. These results provide additional evidence that αP-Thr recognized EGF receptor, not due to the cross-reactivity with P-Tyr, but due to the reactivity with P-Thr.

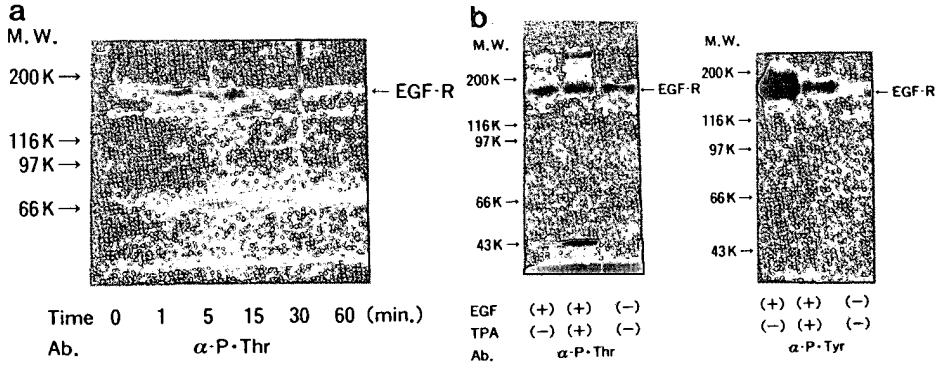

Fig.4a) Immunoblotting of TPA-stimulated phosphoproteins in A431 cells:A431 cells were stimulated with or without 10^{-8}M TPA for the indicated time and boiled in Laemmli's sample buffer. The proteins were run on SDS-PAGE and immunoblotted with αP-Thr.

b) Immunoblotting of EGF-induced phosphoproteins in A431 cells pretreated with TPA A431 cells, pretreated with or without 10^{-8}M TPA for 15 min at 37°C, were stimulated with 20 ng/ml EGF for 5 min and boiled in Laemmli's sample buffer. The proteins were run on SDS-PAGE and immunoblotted with αP-Thr or αP-Tyr.

Recently, a few papers have been published on the preparation of antibodies toward phosphoserine and phosphothreonine (14,15). Unlike these previously reported methods, we used BSA conjugates of phosphothreonine or phosphoserine as antigens, and purified the antibodies with phosphothreonine or phosphoserine affinity column. To obtain αP-Thr, the phosphothreonine column was eluted first with phosphoserine and then with phosphothreonine, while for αP-Ser, the phosphoserine column was eluted with phosphothreonine and then with phosphoserine. These procedures may have enabled us to obtain antibodies with high specificity. These antibodies were capable to immunodetect some, but not all, proteins which were phosphorylated on serine or threonine residues. Some phosphoproteins were immunoblotted by the antibodies but not immunoprecipitated. These data suggest that some proteins were recognized by the antibodies only when they exist either as intact molecules or as denatured molecules.

To our knowledge, this is the first report on antibodies which recognize insulin- or EGF-stimulated serine or threonine phosphoproteins, in particular, insulin receptors. The antibodies will certainly provide a good tool in the evaluation of insulin and EGF receptor's phosphorylation and the detection of novel kinases and substrates in the signal transduction as the antibody against phosphotyrosine facilitated the discovery of IRS-1 (16).

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